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Selected presentations given at this conference, held in September 1987, are reviewed. Topics are: cell harvesting and disruption, adsorption and chromatography, analytic techniques and process control, liquid-liquid extraction, and membrane extraction. Contents of this report include: —

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INTERNATIONAL CONFERENCE ON SEPARATIONS FOR BIOTECHNOLOGY: READING, UK

1 INTRODUCTION

This conference, sponsored by the Solvent Extraction and Ion Exchange Group and the Biotechnology Group of the Society of Chemical Industry, was held at the University of Reading, UK, from 15 through 18 September 1987. Of the total of 181 participants, 68 percent represented industrial organizations with the balance from academic institutions. Although the majority of attendees were from the UK, an appreciable number also came from 10 West European countries as well as the US and New Zealand.

The program format of this focused and informative conference included plenary lectures, oral presentations and poster sessions on the following topics:

- Cell harvesting and disruption
- Analytical techniques and process control
- Adsorption and chromatography
- Liquid-liquid extraction
- Product containment and safety
- Membrane processes.

There is increasing commercial and industrial potential for the growing range of products from the "new biotechnology," and much emphasis has been placed on the production processes for substances with biological activity. However, very little has hitherto been available on the important topic of downstream processing, covering the principles involved in recovering a product in a usable form. Thus, this conference has focused on this aspect and the presentations describe many of the developing techniques for both new and established products--for example, enzymes, antimicrobial agents, and hormones. In addition, methods were described for separation of a required product from its producing organism and extraneous impurities.

Since there was a great deal of material presented at this intensive conference, only selected topics will be reviewed in this report.

2 CELL HARVESTING AND DISRUPTION

Recovery of Cells from Fermentation Broths

The subject of recent and novel developments in the recovery of cells from fermentation broths was discussed by P.N. Whittington (Biotechnology and Separations Division, Warren Spring Laboratory, Stevenage, UK). Whittington described six novel separation technologies (dielectrophoresis, electrokinetic, ultrasonic, liquid-liquid partitioning, magnetic, and foam separation) and their application to primary bioprocessing. The envisaged advantages and disadvantages of applying each technology to primary bioseparation were also discussed by Whittington.

The increasing use of fermentation processes to manufacture specific products, such as enzymes and pharmaceuticals, is placing increasing demands on the processes used for product separation, according to Whittington. One consistent need within bioprocessing is for effective methods of treating the products of fermentation--either to recover intact cells, which can subsequently be disrupted to recover intracellular products, or to separate cells (or cell debris) to obtain a clarified liquid. In addition, there is often the need to recover cells selectively from other bioparticulates. There are many problems associated with the recovery of cells and cell debris and other separative activities associated with fermenter broths. The complex biochemistry, high viscosities and severe fouling characteristics of cells and cell debris suspensions contribute to the difficulty of the separation. The small size and low density of cells and biocolloids, together with the sometimes low concentrations of the fragile products, add to the problems.

In searching for alternative methods of separation, various properties of cells can be exploited including size, density, hydrophobicity, polarizability, and electric and magnetic charges. The exploitation of some of these properties by the six novel separation processes mentioned above was evaluated by Whittington and his group as part of a collaborative project on downstream processing funded by industry and the UK Department of Trade and Industry.

Electrophoresis. Under the influence of an electric field both electrophoresis (the transport of a charged surface relative to a stationary liquid) and electro-osmosis (the transport of a liquid relative to an immobile charged surface) will occur. These effects have the greatest significance for fine solids for which the electrical fields outweigh gravitational forces. This means that the solids must be evenly dispersed to take full advantage of electrophoretic effects. Not all of the electrical energy supplied is used for the transport of particles or solvent, and inherent limitations are introduced by this energy insufficiency, according to Whittington. As well as electrical heating of the slurry, electrode reactions will also occur. Both effects may lead to denaturation of biological products. The applied potential may also generate undesirable convective currents within the feed.

According to Whittington, electrophoretic processes are established techniques only for the dewatering of fine clays and latexes. To date, only a small amount of work has demonstrated the technical possibilities of application to the treatment of biological particulates, although in principle, this should be possible, especially for the smaller particulates. Although the majority of biological particulates have suitable charge properties, in many cases the conductivity of the medium is a severe limitation on economic operation. This limitation will be less severe when the solid product is being washed and there may be significant opportunities, from both increased energy efficiency and product quality, for dewatering within the final solid-liquid separation and drying stages. Electro-augmented filtration, especially crossflow filtration, also appears to offer economic advantages, according to Whittington. There may be scope for electrophoretic cleaning of particulate-fouled membranes. Apart from these applications, however, the future economic impact of this technology is likely to be low, as stated by Whittington.

Dielectrophoresis. The movement of charged or neutral particles which can be electrically polarized in a nonuniform electrical field. Charge polarization will occur in both alternating and direct nonuniform fields; however, particle movement in the former is due solely to dielectrophoresis. In direct current fields electrophoretic forces also exist, and electrolysis can also occur. In a nonuniform field the polarized particle experiences a stronger attractive force to the area of highest field strength and will move in that direction irrespective of the polarity of the electrodes. Dielectrophoresis has been exploited for the recovery of inorganic particles from liquids of low conductivity, and high gradient dielectrophoretic separations can be as effective as mechanical filtration, according to Whittington. Due to the electrical double layer at the surface of biocolloids, they are very polarizable and thus are susceptible to dielectrophoresis due to their high dielectric constants. Despite this, the dielectrophoretic recovery of biocolloids from conductive broths has not been exploited, according to Whittington, and most practical demonstrations have been undertaken at the laboratory scale. Table 1 provides a summary of some of the published data at this scale of operation where very small quantities (<0.001 m^3) of cell suspensions have been processed. Some of the researchers listed in Table 1 have demonstrated the selectivity of dielectrophoresis, and this is where future commercial applications may prove to be the most attractive in separating different cell types, according to Whittington. In addition, the separation of cells in different metabolic states but of the same cell type has also been demonstrated. One important application which has not yet been proven is the selective recovery of intracellular product biocolloids from cell debris suspensions.

Table 1
Laboratory-scale dielectrophoresis of biocolloids

Biocolloid Type	Comments	References
Euglena cells	alignment and clumping of cells	Griffin and Stowell 1966
Human erythrocytes	alignment and bunching	Griffin 1970
Yeast	collection	Pohl 1971
Liver mitochondria	collection	Pauley et al., 1960
Amoeba	collection	Friend et al., 1975
E. Coli	collection	Chen 1972
Ps. aeruginosa		
B. cereus		
B. megaterium		
S. aureus		
Ps. fluorescens		
Mycoplasma	collection	Carstensen et al., 1971
Flavobacteria	collection	Wiley, 1970

Whittington stated that the primary technical barrier to the development of dielectrophoretic phenomena for the processing of biosystems (cell and other biocolloidal suspensions) remains the problem posed by the relatively high conductivities (0.01 mho cm^{-1}) of the process streams and the subsequent reduction in the performance of dielectrophoresis. Research will be needed to control these aspects before the full potential of this technique can be exploited.

Ultrasonics. Sound waves have been applied for either pretreating feeds prior to separation by established techniques, or as an integral part of a separation system. They have also been applied to enhance drying, precipitation, and emulsification processes. Ultrasonics--sound waves above 16 kHz frequency--form the majority of experimental applications in this area of acoustic techniques. However, there are applications which use audible frequencies. For example, low-frequency conditioning of soya protein precipitate using a dual source generator operated at two frequencies, producing a resultant beat frequency has been reported. The most relevant application for ultrasonics, according to Whittington, is probably dewatering. Ultrasonic transducers have been positioned in the air, in the liquid, or directly on a filter to improve gravity settling, filtration, and centrifuge operations at a laboratory or small pilot scale. Treatment of biological materials at present has been restricted to gravity settling of sewage sludge. For low-frequency sonics, biological applications studies have included precipitation and centrifugation of both blood fractions and soya protein.

The major unknown with both ultrasonics and sonics is whether the energy costs for a power transducer exceed the benefits from the increased performance of the separator. Efficiencies of conventional separators for submicroscopic particles such as cell debris are comparatively low. Whittington considers it likely therefore that there is considerable scope for improving these efficiencies by applying sound waves to aggregate the particles. Unfortunately, most of the research has dealt with much larger particles, and therefore there is a need to study ultrasonic submicron particle aggregation.

Two-Phase Aqueous Extraction. Due to the tendency for many biochemical products to denature on contact with the oil phase used in conventional liquid-liquid extraction, much research work has gone into the development of two-phase aqueous systems, in which the presence of polymers or salts in the phases leads to immiscibility. Polymers can be selected which have no deleterious effect on the cells and actually exert a protective action. The viability of recovered cells is excellent and the process can be operated at room temperature with a significant saving in cooling costs compared with some alternative processes. A feature of two-phase aqueous extraction is the ease of scale-up, with processes successfully scaled up from 10 ml to 250 to 400 liters, a factor of 25 to 40,000. The immiscibility between the two aqueous phases results from differential partition of a polymer--for example, polyethylene glycol (PEG), dextran or salt--between the two phases. Cells, particles, and macromolecules partition between the phases with partition coefficients between 0.01 to 100 (cells/particles) and 0.01 to 10 (proteins). The interface has different properties from either of the bulk phases, and in certain circumstances this can be exploited to recover cells/particles, according to Whittington.

Cell partition is a function of hydrophobicity, cell surface charge, and lipid properties of the cell membrane. Partition behavior is determined by the concentration and molecular weight of the polymers used, the type and concentration of the salts present, the pH and ionic strength of the phases, and temperature. There are thus opportunities for optimizing partition behavior, and these have been exploited on a laboratory scale for separation of cell populations and up to the pilot scale for direct recovery of enzyme, glucose, and ethanol from fermentation broths. Particular difficulties arise in processing two-phase aqueous systems because of their low interfacial tension, high viscosity of the phases, and low density difference between the phases. Mass transfer in contactors is generally rapid, in part because of the small droplet size and large interfacial area, but phase separation rates are low. While gravity separation is usually satisfactory with PEG-salt systems, centrifugal separation is usually required in PEG-dextran systems to avoid long separation times.

The most promising area of application of two-phase aqueous systems to downstream processing is in the area of selective recovery of soluble product from a broth containing debris, according to Whittington. A number of enzymes have been extracted from broths containing 20 to 25 percent wet cell mass (broken cells) at room temperature by the research group at the Institute of Biotechnology in Braunschweig, West Germany. The extraction has been successfully achieved from homogenates of prokaryotic and eukaryotic microorganisms as well as from gram positive and gram negative bacteria. Other examples are the extraction of beta galactosidase from *E.coli* and the recovery of glucose from enzymatic conversion of starch. Cost comparisons have suggested that the two-phase aqueous extraction could be competitive with conventional techniques.

According to Whittington, two-phase extraction is a very active research area both for separation of cells, especially in the biomedical fields, and in downstream processing. It appears that workers in both branches of the field are beginning to come together. Further improvements in the process are anticipated from this synergy, both from an understanding of the fundamental biochemistry of the process and of the chemical engineering of liquid-liquid contact and separation in high-viscosity/low-interfacial tension systems.

<u>High Gradient Magnetic Separation Processes.</u> In the mineral processing industry, there are numerous large-scale recovery systems in operation which can rapidly recover magnetic particles selectively from dense slurries. Since the development of high gradient magnetic separation (HGMS) in the mid-1970's, the applicability of the technique has broadened to include the recovery of smaller and/or more weakly magnetic particles and even nonmagnetic biological particles. Large-scale recovery of biological particles by HGMS has to date involved magnetic, seeded flocculation of the particles to increase both their

particle size and magnetic moment. More selective magnetic labeling is possible by several means: bioaffinity labeling to a magnetic particle/gel microsphere or ferritin, metal ion accumulation, and manipulation of cellular heme proteins.

High gradients are produced by insertion of sharp points, edges, or wires or a magnetizable material, (for example, steel wool) into the main field, thereby distorting the applied magnetic field and generating strong local field gradients. The collecting force acting on a particle in a magnetic field is proportional to the magnetic contrast, the magnetic field strength, gradient, and its volume. Typical large-scale nonselective applications include effluent and water purification. More selectively, red and white blood cells can be recovered from plasma and magnetic particles linked to microbial cells, cancer cells and enzymes. Provided the all-important labeling step is accomplished, Whittington thinks that there are many applications for this technology in bioseparations.

Foam Separation Processes. This technology exploits the phenomenon that certain materials, both particulate and soluble, accumulate at gas-liquid interfaces, forming a concentrated interfacial layer. Such surface active particles and molecules (for example, proteins and lipids) will attach to rising gas bubbles in the liquid and collect in the surface foam layer. The efficiency of flotation for fine particles is determined by two factors: (1) the collision frequency and (2) the attachment efficiency. Collision efficiency is determined by the size and density of the particle and hydrodynamic forces in the bubble column. Attachment is determined by the particle's surface chemistry, notably its charge and hydrophobicity.

Mineral processors make considerable use of foam separations, using collector surfactants to control the surface chemistry of the particles they wish to recover. Bioparticles such as cells and subcellular colloids are more difficult to float due to their small size, small density and usually hydrophilic character. Currently, dissolved air floc flotation systems are most commonly used commercially for effluent treatment and protein recovery. Researchers have adopted several approaches to improve the collection efficiency of this technique, including flocculation to increase particle size, reducing bubble size, and cell surface conditioning. More recently, researchers have avoided using costly (and possibly harmful) conditioning reagents and have optimized other parameters (for example, cultivation conditions, float cell geometry, etc.). Whittington thinks, however, that foam separations require extensive development before the likely technical and economic gains from their use in bioprocessing are realized.

Of the novel separation techniques reviewed above, Whittington and his coworkers view dielectrophoresis as a particularly promising novel technology and are currently evaluating its application to bioprocessing. Ultasonics also rates highly and some potential has been demonstrated for liquid-liquid and magnetic separations. There is, however, limited scope for the use of electrophoresis in primary separations, and any application of foam separations would require extensive development, according to Whittington.

Biochemical Engineering Aspects of Cell Disruption

This subject was discussed by E. Keshavarz (Department of Chemical and Biochemical Engineering, University College, London, UK). The recovery of intracellular materials, such as proteins and enzymes from microorganisms, has gained a new momentum over the past decade as a result of novel commercial applications in the food and pharmaceutical industries and due to the prospects of further product developments by genetic engineering. The design and operation of large-scale extraction and isolation systems for intracellular proteins and enzymes have been constrained by the denaturation and the loss of activity of products during processing, according to Keshavarz. Better understanding of each step from product formation in fermenters to cell breakage, separation, and purification has been shown to improve the development of industrial techniques. Cell disruption constitutes the first stage in the isolation of intracellular materials. It is a crucial step in downstream processing because any damage to the product at this initial phase will invalidate subsequent design and make operational refinement to other plant equipment irrelevant. On the other hand, high disruption yield will allow, according to Keshavarz, more flexibility in the subsequent treatment of the product.

While there are many specific examples of chemical or lytic-based disruption for the release of intracellular proteins, it is the mechanical disruption methods which have found general application on the pilot and industrial scales. When processing heat-labile materials which are also prone to microbial and proteolytic degradation, it is necessary, according to Keshavarz, to use low residence time and continuous operations. Furthermore, especially with the advent of modern recombinant DNA (rDNA) techniques for the manufacture of mammalian proteins in microorganisms, it is necessary to operate cell disruption equipment in a contained fashion with the possibility of in-place cleaning and sterilization. Two disruption techniques available to meet these requirements, according to Keshavarz, are high-speed bead mills and high-pressure homogenizers. She discussed the operating characteristics of these techniques with respect to the overall efficiency of protein and enzyme release as well as the effect of cell choice and growth conditions on the success of the disruption process.

High-Speed Bead Mills. Originally, bead mills were used in industry for fine grinding and dispersion of dyestuffs and pigments. The design of bead mills varies with the size of the unit and the manufacturer. Mills consist of either a vertical or horizontal grinding chamber containing rotating discs or impellers mounted, concentrically or off-centered, on a motor-driven shaft. The grinding action is provided by beads typically occupying 80 to 85 percent of the free working volume of the chamber. The units must be equipped with high-capacity cooling systems for processing temperature-sensitive materials. Horizontal units are generally preferred for cell disruption because the grinding action in vertical mills is reduced due to fluidizing effects of the upward flow on the beads.

It has been reported, according to Keshavarz, that, for a range of bead diameters studied (0.5 to 2.8 mm), the smaller beads were more effective. For a 30-percent weight/volume packed yeast slurry small beads were favored while above a concentration of 60 percent, their advantage was reduced. The variation of disruption efficiency with yeast concentration was found to be similar for beads of different size and no special effect of large beads on thick yeast suspensions was observed. Also, it appears that for yeast cells, the location of a desired enzyme within a cell can influence the optimum bead size. Larger beads can be used to recover enzymes located in the periplasmic space and smaller ones to release the cytoplasmic material. Thus, it appears that smaller beads are more effective in achieving complete cell disintegration. The disintegration of other microorganisms such as several varieties of bacteria and filamentous fungi has been reported in the literature.

First-order kinetics have been observed for the batch-wise disruption of yeast cells, where the rate of disruption as measured by the rate of soluble protein release was directly proportional to the amount of unreleased protein. For a first-order disruption process it would be expected that the rate constant is independent of cell concentration. This has been found to be the case for a 5-liter horizontal bead mill for high yeast concentrations (greater than 30-percent weight/volume packed yeast) when disrupted using stainless steel discs where the open disc area is very small (called closed discs). In contrast, for polyurethane discs with a large open area, "open discs," the rate constant decreased significantly with increased yeast concentration. It was also found that increased disc tip speed led to virtually no increase in the rate constant for the "open discs" while for the "closed discs" the disruption rate constant increased with greater disc speed. In all cases studied, it appears that the "open discs" gave greater disruption rate constants than was observed for the "closed discs." Operation with greater yeast cell concentrations (75-percent weight/volume packed yeast) leads to an eightfold increase in viscocity. For the bead mill operating at low disc speed and hence no backflow, greater viscosities of the flowing suspension will tend to a decreased attrition rate in the region of the disc, especially for the discs of "open design." Hence, higher viscosities resulting from operation at increased cell concentration are likely to lead to lower overall rates of disruption, according to Keshavarz.

<u>High-Pressure Homogenizers.</u> High-pressure homogenization is the most widely used method for large-scale cell disruption. The equipment consists of a high-pressure, reciprocating, positive-displacement pump with one or more adjustable, restricted orifice valves. All commercial models operate on the same principle and are distinguished by their capacity, the type of homogenizer valve, pressure range, drive mechanism, and number of pistons. Cell suspension is drawn through a check valve into the pump cylinder and on return of the piston is forced through the discharge cylinder and through the discharge valve assembly. The discharge pressure is controlled using a spring-loaded valve rod which positions the valve in relation to the valve seat. During discharge the suspension passes between the valve and its seat and impinges on an impact ring. The pressure is shown on a gauge mounted on top of the cylinder. In the case of a single-piston homogenizer, fluctuations in pressure measurements are reduced by a dampener assembly. In the past, homogenizers were supplied with either a flat-edge "standard" or a knife-edge "cell rupture" valve unit. The latter model is now superseded by a modified knife-edge valve unit--the "cell disruption" valve (see Figure 1).

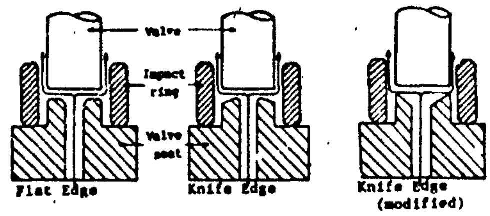

Figure 1. Diagram of homogenizing valve units.

Several operating parameters have been identified as affecting the performance of a high-pressure homogenizer such as pressure, number of passes, temperature, and cell concentration. It has been found that disruption followed first-order kinetics with respect to the number of passes and can be described by a general equation which takes into account all the parameters for disruption. Valve design also plays an important role in the efficacy of cell disruption. APV-Gaulin Incorporated has investigated the effects of homogenizing valve geometry on the cell disruption efficiency. Results were monitored by measuring the percentage of total available protein released after disrupting a 10-percent dry weight slurry of baker's yeast. Five valve designs were tested with various pressures. The results indicated that a knife-edge "cell disruption" unit was superior to other valve designs (Figure 1). This company also used ceramic materials, which are more resistant to wear.

<u>Cell Characteristics.</u> Conditions of cell growth have been found to have a major effect on disruption kinetics, according to Keshavarz. Even in the case of *Saccharomyces cerevisiae* differences have been observed between disruption rates of baker's yeast and spent brewer's yeast. Results obtained for the disruption of *E.coli* cells clearly indicated that batch cultures grown on a synthetic medium were easier to disrupt than those grown on a complex medium. Also, it has been shown that cells grown at a higher specific growth rate were easier to disrupt than cells grown at a lower specific growth rate. Similarly, cells harvested during the log phase of growth were more

Table 2
Protein release from microorganisms in a high-pressure homogenizer

Microorganism	Rate constant, K
Pseudomonas putida	0.41
Escherichia coli	0.39
Bacillus brevis	0.28
Saccharomyces cerevisiae	0.23
Nocardia rhodochrous	0.0085

susceptible to homogenization than those from the stationary phase. Cell wall structure also plays a role in the efficacy of disruption. It has been shown that gram negative bacteria are easier to disrupt than gram positive bacteria which, in turn, are easier to disrupt than yeasts (see Table 2). Fungal walls are classified as more resistant to disruption.

Keshavarz stated that when considering the efficiency of cell disruption it is necessary to take into account the yield of the desired product in an active form--for example, for intracellular enzymes or for therapeutic proteins from genetically engineered organisms. Due to the diverse nature of the intracellular products obtained from microorganisms and the considerable variation in their stability it is not possible to provide general quantitative guidelines. For example, there may be loss of activity due to shear for enzyme-membrane complexes whereas soluble, globular, intracellular proteins are not normally damaged by shear, but by shear-associated effects, as, for example, in the presence of air-liquid interfaces. Thus, care is needed in the design of homogenizers to prevent air entrainment. Another source of yield loss is thermal inactivation. Much of the power input into bead mills or high-pressure homogenizers is translated directly into thermal energy with consequent large temperature increases. Scale-up of the bead mills leads to proportionally less surface area available for cooling and consequently greater temperature rise. Therefore, high throughput operation with interstage cooling is required. However, Keshavarz thinks that there is now a reasonably good description of the factors influencing performance of bead mills and high-pressure homogenizers. Thus, she suggests that scale-up can be predicted and that the release of water soluble proteins and enzymes can be achieved without significant damage using either of these two methods of cell disruption.

Biosurface Properties--Primary Separation

Biosurface properties and their significance for primary separation were discussed by S.R. Warne (Biotechnology and Separations Division, Warren Spring Laboratory, Stevenage, UK). Warne spoke about the forces which maintain bacterial walls in a dispersed state and described the mechanisms by which they are overcome during the formation of cell aggregates. Such aggregation mechanisms involve a number of physico-chemical properties of the cell surface. Warne said that insofar as these properties are attributable to specific molecules at the cell surface they may be open to genetic control. He described one example of a bacterial strain which exhibits autoaggregation. This example illustrates the way in which alterations at the genetic level can alter the composition and physico-chemical properties of the cell surface and how this in turn affects the ability to autoaggregate.

In biotechnology the formation of cell aggregates is highly advantageous for the optimization of the primary separation processes by which bacterial cells are separated from their growth medium, according to Warne. This is because the efficiency of the separation techniques is related to particle size. Since cell aggregation is inexorably linked to cell surface properties it is clear that an understanding of cell surface characteristics, and how to control them, can help to improve both the efficiency and economics of downstream processing. Up until now the main way in which surface properties have been controlled within the context of primary separation has been by addition of chemicals to the cell broth. However, according to Warne, the advent of genetic engineering technology has opened up the possibility of controlling those genes which encode surface components in order to specifically alter the composition and physico-chemical properties of the cell surface.

Although there are methods by which cell aggregation can be artificially induced by the addition of flocculants or metal ions there is increasing interest, according to Warne, in the utilization of natural aggregation phenomena in biotechnology. One advantage of such natural aggregation is the avoidance of contamination by flocculants in subsequent downstream processing following the primary separation stage. There is relatively little known about the molecular basis of natural aggregation and so, in order to obtain a more complete understanding of the mechanisms involved, a particular example of natural clump formation is being studied by Warne and his group. The aggregation phenomenon being investigated is known as fluffing and is exhibited by some strains of *E. coli* K12. This fluffing phenomenon involves the formation of cell aggregates, known as fluffs, which precipitate from unshaken cultures. The control of this ability to fluff has been ascribed to a gene designated *flu*. The location of the *flu* gene on the *E. coli* chromosome has been determined. It lies between the histidine operon *(his)* and the *sup D* locus on the *E. coli* linkage map. Since the molecular mechanism of fluffing must involve one or more specific surface components, the aim of the research by Warne and his group is to demonstrate the way in which changes at the genetic level affect the composition of the cell surface and how this in turn affects the physico-chemical properties of the cell.

The fluffing phenomenon is complicated by the fact that strains which exhibit fluffing generally exist in two interconvertible forms known as flu^- and flu^+. The switching between these two forms is such that cultures of both flu^- and flu^+ strains grown from single colonies contain 1 to 10 percent revertants. There is a marked difference in colony morphology between flu^+ and flu^- variants with flu^+ colonies being glossy and smooth in texture and circular in shape while flu^- colonies are rough and irregular. Thus, when a culture grown from a single colony is plated out on solid medium the majority of the colonies are of the same type of morphology as the initial colony while a few show the other type of morphology. Of the two variant forms it is only the flu^- variant which forms the cell aggregates which result in sedimentation when the shaking of a liquid culture grown from a single colony is stopped. In contrast, the flu^+ variant does not form cell aggregates and remains in a stable homogeneous suspension.

The cell aggregation which characterizes the flu^- genotype can be seen when a sample of shaken culture is examined under a microscope. Small cell clusters are clearly visible. If the culture is then left to stand at room temperature the clusters grow into small grains visible to the naked eye and eventually form the large fluffs which precipitate to form a cell pellet. It appears that the interactions which bind the fluffs together are weak since they can be readily dispersed by agitation.

It is thought that the switch of a strain from one *flu* variant form to the other is accompanied by an alteration of the ability of the bacteria to form hairlike structures known as type

I pili (fimbriae) on their cell surface. Evidence for this has come from preliminary studies of *flu*$^+$ and *flu*$^-$ using electron microscopy and from tests of the ability of *flu*$^+$ and *flu*$^-$ cells to agglutinate horse erythrocytes. Despite this relationship between piliation and the state of the *flu* gene, Warne said that it is important to note that *flu* variation has been observed in a pili strain that lacks the genes required for pili formation. It seems, therefore, that in the case of *flu*-mediated aggregation the presence or absence of pili is not a primary determinant of the ability of cells to form cell clumps. Thus, although the genes involved in fluffing may exert some form of direct or indirect control over piliation it seems that the molecular mechanism of *flu* variation primarily involves the regulation of the production of another cell surface component, according to Warne.

In addition to the *flu* gene another region of the *E. coli* chromosome has been implicated in the *flu* phenomenon. It has been found that deletion of a small segment of the chromosome between the arginine operon (*arg*) and the *btu B* gene gives rise to a mutant that is permanently in an aggregating form--i.e., such strains do not form any *flu*$^+$ variants. Since this region of the chromosome appears to be involved in the *flu* phenomenon it has been postulated that the chromosome contains a second gene, designated *flu B*, which acts in conjunction with the *flu* gene itself to mediate cell aggregation.

Preliminary studies were carried out by Warne and coworkers to characterize the differences in the physico-chemical properties of the cell surface which result from the deletion of the *flu B* gene. The most interesting point to emerge, to date, is that cells in which the *flu B* gene is deleted (*flu B*$^-$) have a significantly reduced surface charge (zeta potential) as compared with *flu B*$^+$ cells. Warne said that this may indicate that a charge neutralization mechanism is involved, but further work will be necessary to confirm this.

<u>Use of Genetic Manipulation to Study the *Flu* Phenomenon.</u> Warne stated that the fact that the methodologies available for molecular biology research are more highly developed in *E. coli* than in any other bacterium has made *flu* strains a suitable model system in which to study the genetic basis of autoaggregation. The studies carried out by Warne and his group have focused on the *flu* gene. A gene bank representing the DNA present in a strain exhibiting switching between *flu*$^+$ and *flu*$^-$ forms has been constructed. From this gene bank a cosmid clone which restores normal *flu* variation to a strain carrying the *flu B*$^-$ deletion has been obtained. The chromosomal insert present in this plasmid must therefore contain the *flu B* gene.

According to Warne the cloning of the *flu B* gene opens up a number of avenues for future research. Firstly, it will enable the group to identify the *flu B* gene product and to determine whether it is itself a cell surface protein, a regulatory protein, or an enzyme involved in the synthesis of a surface polysaccharide or lipopolysaccharide. Secondly, it will be possible to use the *in vivo* operon fusion technique to link the expression signals of the *flu B* gene to the *lac z* structural gene which encodes beta galactosidase. The ease of assaying for galatosidase will then make it possible to readily determine whether or not the *flu* variation results from the switching on and off of *flu B* transcription. Two bacterial surface components that are under a similar type of control to *flu* variation are type I pili in *E. coli* and flagella in *Salmonella typhimurium*. In both of these cases the variable control has been shown to result from the inversion of a small DNA segment upstream from the structural gene which results in an alternating orientation of the promoter of this gene. Warne thinks that studies of *flu* gene regulation may well reveal that a similar mechanism operates in this case. Thirdly, by using a suitable vector, Warne suggests that it may be possible to overproduce the gene product of *flu B* and determine the effect of

Table 3
Bonded phase selectivities of some chromatography ligands

Ligand	Structural Features Selected
Antibodies	Binding to immunoactive protein domains
Lectins	Adsorption to carbohydrates in glycoproteins
Substrate analoques	Reaction with enzyme active or cofactor binding sites
Dyes	Binding to empirically found domains, e.g., Cibacron blue and pyridine nucleotide sites
Metal ions	Chelation to histidine
Thiols	Covalent binding to cysteine
Cations	Ionic binding to lysine, arginine and histidine
Anions	Ionic binding to glutamate and aspartate
Phenyls, C3-C8-aliphatics	Hydrophobic interaction with non-polar amino acids
High density C12-C18-aliphatics	Reverse phase interaction with non-polar amino acids

such a perturbation on the ability of the cells to aggregate.

3 ADSORPTION AND CHROMATOGRAPHY

The isolation and purification of proteins using preparative HPLC was discussed by S.J. Brewer (Corporate Research and Development, Monsanto Chemical Company, St. Louis, Missouri). High-performance liquid chromatography (HPLC) is the result of improvements in chromatography and solvent delivery systems. Brewer described the use of HPLC to prepare highly purified beta- and gamma-interferons, albumin, somatotropin, trypsin, and beta-carotene in milligram to multigram quantities. Brewer said that these examples support the thesis that chromatography media purify proteins by a combination of selective adsorption (selectivity) and dynamic partitioning. High-performance solvent delivery systems and medium selectivity, whether rationally designed or empirically discovered were used in all of the above examples. Only when selectivity was not sufficient to resolve impurities was the dynamic partitioning of proteins between the mobile phase and the bonded phase used to achieve purification. In this mode, the carefully controlled physical properties typical of analytical-grade chromatography medium were required. Proteins purified by HPLC with media of one selectivity showed microheterogeneity when analyzed using different media. Therefore, Brewer emphasized that it is necessary to use HPLC media of different selectivities to prepare ultrapure proteins.

Liquid chromatography separations are achieved by the selective adsorption and differential partition of solutes between a fluid mobile phase and a bonded phase. With size exclusion chromatography, the bonded phase has no selectivity. Proteins separate by dynamic diffusion into an inert porous particle. The dynamic properties are dependent on the overall size and shape of the protein and pore as well as on the mobile phase linear velocity and flow pattern. Most preparative chromatography media, however, use bonded phases which are covalently linked to the surface of a rigid matrix and adsorb proteins. Selectivity is a function of a protein-binding ligand covalently attached to the matrix (see Table 3). Affinity chromatography uses a ligand of such high selectivity that only a specific protein is adsorbed. After washing away nonadsorbed impurities, a highly purified protein is eluted. When bonded phases with intermediate selectivity are used, the

Table 4
Physical properties of HPLC media for resolution by dynamic partitioning

Property	Description
Surface area per unit weight	High, to maximize bonded phase/solute interactions
Achieved by:	High porosity and/or small particle size
Size heterogeneity	Low, typically within 20 percent of mean, to ensure bonded phase homogeneity
Achieved by:	Sizing of particles or controlled polymerization (monobeads)
Shape distribution	Approaching spherical to allow regular flow characteristics and minimize peak broadening
Achieved by:	Monodispersion
Stability	High, mechanical strength for fast flow rates and chemical stability for multiple recycles
Achieved by:	Use of silica or hydrophilic polymers

mobile phase composition can be adjusted and proteins resolved by differences in their dynamic partition rates. Thus affinity and size exclusion chromatography are at the extremities of a continuum of methods which separate by a combination of selectivity and dynamic partitioning. When these parameters are optimized and combined with a quality-engineered solvent delivery system, liquid chromatography attains the performance characteristics which define HPLC, according to Brewer. Selectivity of the chromatography medium is defined by the matrix and ligand which form the bonded phase. Affinity chromatography uses a bonded phase designed to adsorb a highly defined area of a protein's surface. For example, a bonded phase made from a competitive inhibitor of an enzyme will adsorb by binding to the protein's active site. Brewer said that it now appears that all adsorption chromatography involves a similar limited contact between the bonded phase and the protein's surface. Thus adsorption and affinity chromatography are mechanistically similar. Affinity chromatography is strategically different in that selectivity is rationally designed while in other methods the selectivity must be empirically determined. The selectivity of such a chromatographic medium is unique because it depends on the ligand, its purity, chemistry and the density of immobilization as well as on the matrix. Therefore, according to Brewer, a primary aspect of HPLC medium production must be the control of this complex chemistry so that selectivities are reproducible.

When solutes are separated using dynamic partitioning, the resolution of chromatography columns should be proportional to the number of plates, according to Brewer. HPLC columns with many thousands of theoretical plates are capable of resolving proteins which have very small differences in partition rates. This requires chromatography media which achieve a bonded phase area with small particles of uniform size rather than with large porous particles (Table 4). Small particles are better than large because the bonded phase is more uniform. These small uniform particles are bought at the cost of high back-pressure and great expense, neither of which is favorable for preparative HPLC. Therefore, the large porous particles used in most preparative procedures sacrifice resolution in order to reduce both cost and back pressure. However, if the desired resolution cannot be achieved, multiple runs with analytical-grade HPLC chromatography media may be required.

Brewer and his group prepared mouse ascites monoclonal antibody (Mab) against beta-interferon. The Mab was purified using protein A and immobilized on activated Sepharose at 7 mg/ml and on activated 10-micron silica at 15 mg/ml. Recombinant DNA-derived interferon produced by cultures of *E. coli* was loaded onto columns equilibrated with pH 7.0 phosphate buffered saline (PBS), washed extensively with equilibrating buffer then with 10 percent propylene glycol, 1 M NaCl, 1 percent Tween, and PBS buffer and eluted with 50 mM citric acid. A Gilson HPLC system was used for the silica-immobilized antibody which was packed in an 8×70-mm column and run at 1 ml/min. Antibody was measured using an ELISA assay. In this preparation affinity chromataography was combined with HPLC to combine the speed and resolving power of HPLC with the selectivity of affinity chromatography.

Recombinant DNA technology allows the rational engineering of proteins to achieve high selectivity for a specific bonded phase, according to Brewer. Brewer and his group found that the affinity of γ-interferon for cation exchange chromatography was predictably increased by the addition of C-terminal polyarginine fusion using gene cloning techniques. The affinity of the fusion for a HPLC cation exchanger was such that a 100-fold purification of γ-interferon-polyarginine was obtained in a single step. This was achieved without extensive pretreatment to remove nucleic acids which prevented adsorption to conventional ion-exchangers. A 1-ml analytical HPLC column was used for development of the isolation procedure. The methods was directly scaled to a 200-ml column packed with the analytical medium which enabled the preparation of 50-mg per run of high specific activity γ-interferon for biological studies.

Empirically Determined Selectivity. Brewer said that selectivities of most bonded phases must be empirically determined. Selectivity cannot be related to the physical parameters of the particle (i.e., size, distribution, matrix) but to the complex interactions between the surfaces of the protein and the bonded phase. For example, bovine serum albumin (BSA) is eluted from a reverse phase HPLC column with identical peak symmetry when the mobile phase acetonitrile content was changed from 47 percent to 48 percent irrespective of the gradient shape. Brewer said that the protein must have been irreversibly adsorbed to the bonded phase because 33-bed volumes of 47-percent acetonitrile did not change the elution profile of BSA. This behavior, according to Brewer, is characteristic of a highly selective bonded phase showing no evidence of dynamic partitioning. Since selectivity is unrelated to the media's physical parameters, inexpensive media can be found and is often used for preparative HPLC. A gram of a recombinant DNA-derived somatotropin analog was required to evaluate its *in vivo* biological activity. Brewer said that it was impractical to prepare this quantity of protein using analytical equipment and/or media. Therefore, several matrices were examined until a 50-micron reverse phase silica was found, which, although unable to resolve other components, showed good selectivity for the protein of interest. After three runs, 1 gram of pure protein was obtained without the need for the resolving power of an analytical medium. Brewer stated that separations based on selectivity may not require the use of HPLC medium at all. For example, two somatotropin analogs were resolved using a "low performance" DEAE-cellulose. The proteins co-chormatographed on all other ion exchangers, including small-particle HPLC media. The unique selectivity, however, was maximized using the ability of the HPLC solvent pumping hardware to produce small, carefully controlled changes in conductivity.

Resolution by Selectivity and Dynamic Partitioning. When proteins show the same selectivity for a particular medium, analytical HPLC can separate by exploiting differences in dynamic partitioning, according to Brewer. The resolution of α-from β-trypsin (for use in peptide mapping) relied both on selectivity and dynamic partitioning. The reverse phase HPLC medium used by Brewer and his group showed a high degree of selectivity for the two forms of the enzyme, separating them from other contaminants. A large HPLC column containing this analytical medium was not available and would have been much too expensive for scale-up. Therefore, an automated analytical system was used to prepare protein by 150 repeated runs over 50 hours. However, when a high-cost pharmaceutical is being prepared, large-scale HPLC columns containing analytical media have been used by Brewer and his group. Preparative ion-exchange HPLC was used to resolve recombinant DNA-derived β-urogastrone from a N-terminal lysine analog using a 12-micron 500-ml cation exchange column. The multigram quantities required to support clinical trials were produced using a fully automated analytical HPLC system adapted to deliver the mobile phase at the high flow rates required to drive the column.

Multidimensional Purification and Ultra-Pure Proteins. The protein-surface selectivities of HPLC bonded phases mean that only amino acid substitutions in the contact area will cause chromatographic differences. Urogastrone purified by ion-exchange HPLC had a single peak when analyzed by ion-exchange chromatography and isoelectric focusing, but urogastrone-related species were resolved on analytical reverse phase HPLC. Similarly, a somatotropin purified by reverse phase HPLC showed heterogeneity when analyzed by ion-exchange. Thus, according to Brewer, to prepare ultra-pure proteins, a number of different chromatography media must be used which each interact with different areas of the protein's surface. A good strategy is to use ion-exchange, reverse phase, and size exclusion to purify, based on charged, hydrophobic, and

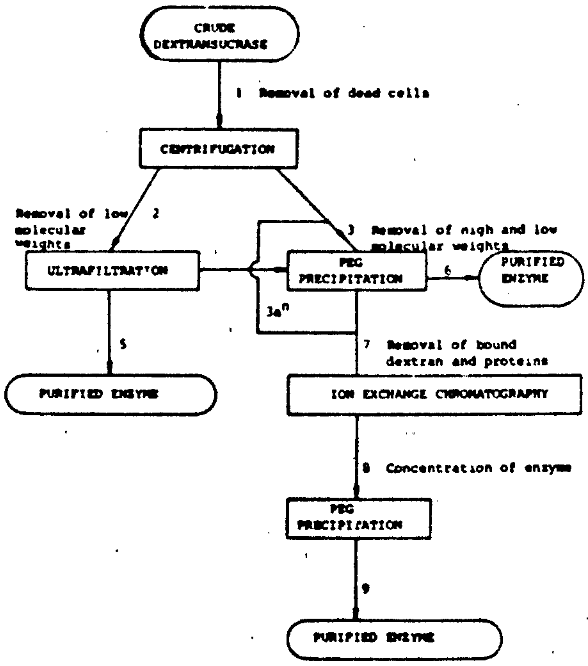

Figure 2. Purification of dextransucrase.

size properties of the protein. However, Brewer thinks that the further development of HPLC may allow such subtle differences to be resolved by partitioning.

Production of Dextran and Fructose

The production of dextran and fructose in a chromatographic reactor-separator was reported by P.E. Barker (Chemical Engineering Department, University of Aston, Birmingham, UK). Dextransucrase-containing fermentation broth produced by the microorganism *Leuconostoc mesenteroides* was purified (see Figure 2) using a variety of methods which included precipitation with polyethylene glycol-6000 (PEG) ion-exchange chromatography and ultrafiltration. The methods used resulted in yields of up to 60 percent of the enzyme. However, it was found that all the purification methods exhibited scale-up problems. The purified enzyme was used to synthesize dextran and fructose in a chromatographic reactor separator. In this reactor, simultaneous reaction and separation resulted in not only pure high-molecular-weight dextran but also the recovery of high-purity fructose. The molecular weight distribution of dextran produced from the chromatographic reactor was higher at the sucrose concentrations used (greater then 15 percent weight/volume) as compared to a conventional batch reactor, according to Barker. The parameters affecting reactor behavior were identified, and it was found that the chromatographic reactor required more enzyme than a conventional batch reactor for a given conversion of sucrose.

Barker pointed out that dextran has many industrial applications and for this reason a significant amount of research has been carried out on its manufacture and properties. Alternative methods of manufacturing dextran include the use of immobilized dextransucrase, but this method, according to Barker, suffers from problems related to stability of immobilized enzyme, viscosity of dextran, and recovery of high-purity fructose. These problems can be lessened if a chromatographic reactor separator, as described above, is used to manufacture dextran and fructose. (There are British and US patent applications for the methods developed by Barker and his group.)

Modeling for Scale-up of Packed-Bed Column

Modeling for scale-up and optimization of packed-bed column in adsorption and chromatography was discussed by I.S. Gosling (BIOSEP, B353, Harwell Laboratory, Oxon, UK). Gosling said that in the scale-up and optimization of adsorption and chromatographic processes there is a need for models that cover the entire process cycle rather than a single step. Many models are only concerned with the adsorption step and simulating breakthrough curves, according to Gosling. However, to model for a unit operation in downstream processing in biotechnology, it is necessary to also model the other steps involved. Thus, Gosling discussed the adsorption of aspartic acid onto the strong anion exchanger Duolite A162 and the subsequent elution of the aspartic acid by a salt solution. Predictions of the performance of a packed-bed adsorption column for loading, washing and elution showed reasonable agreement with experimental results. Gosling also described small-scale batch experiments which provide estimates of the isotherms and kinetic parameters for the model and these were compared with values fitted to experimental data from small columns. Gosling said that differences between these two sets of results indicate a requirement for further research.

The work described by Gosling is part of an on-going program aimed at the adaptation and development of the Harwell FACSIMILE code to the prediction of adsorption and chromatographic processes to provide a tool for performance, scale-up, optimization, and design studies. Thus, Gosling and his group used a simple, lumper parameter, kinetic model in the FACSIMILE code to predict the performance of a small-scale column for the loading, washing, and elution of aspartic acid onto and from Duolite A162 ion-exchange resin. Gosling said that although such a simple model may not perfectly fit the whole process cycle, the agreement over the loading, washing, and elution steps together was sufficient to move to larger systems on which to test the model.

Fractionation of Proteins

The fractionation of proteins at high capacity and high resolution by displacement chromatography was discussed by A.R. Torres (Bio-Fractionations Company, Logan, Utah). Displacement chromatography is based on the direct competition among the adsorbed solvents for column sites. In a classical displacement train, the separated components emerge as contiguous plateaus, each higher in concentration than the preceding one on a mass basis. The component with the lowest column affinity emerges first, followed by components of increasing higher affinities, all pushed along the column at the same rate (isotachic) by the final displacer, which has the highest column affinity. Once the isotachic condition or displacement train is established, the concentrations of the component plateaus are determined by the concentration of the final displacer. Appropriate choice of this concentration leads to the emergence of the components at very high concentrations, according to Torres. The mixing that occurs at the boundaries can be overcome by adding spacing displacers of intermediate affinities to separate the contiguous plateaus. Applications of these principles have been slow in coming because of problems in finding suitable spacers and final displacers. The success and popularity of elution chromatography was another factor, according to Torres. However, displacement chromatography has been applied over the years to the separation of rare earths, amino acids, hydrocarbons, fatty acids, peptides, and proteins. More recently, analytical HPLC columns and equipment have been used for preparative separation of steroids, nucleotides, dipeptides, and proteins by displacement chromatography.

Carboxymethyldextran Displacers. If a soluble polyanion has the proper size and charge distribution, it can be expected to compete and displace a given protein from a binding site on an ion-exchange column, according to Torres. Providing the column has adequate resolving power, a series of such molecules with differing charge will form a displacement train based upon differing ion-exchange affinities for the absorbent. Proteins introduced before or after these spacing displacers, will be positioned on the column according to their abilities to compete for binding sites. The introduction of a displacer having a higher affinity will move the train along the column at a constant rate determined by its concentration. Torres and his coworkers have shown that carboxymethyldextrans (CM-D's) with varying carboxyl contents can serve as displacers on conventional and HPLC columns. Although plateaus are not formed with natural protein samples because of the very large number of different protein species and the lack of sufficient quantity of a single protein, these CM-D displacers have been shown, with simple protein mixtures, to fulfill the criteria established for displacement chromatography. Ampholytes used in isoelectric focusing have also been used to separate proteins on ion-exchange columns. Although such separations have been described as displacement chromatography, Torres said that a displacement mechanism has not yet been demonstrated.

Torres discussed the high capacities obtained in the purification of serum protein on a conventional DEAE-cellulose column and in the resolution of mixtures of model proteins on HPLC ion-exchange columns. Human Gc-globulin is a vitamin D binding protein in serum which has three major as well as many minor phenotypes. Although it has been hard to purify because of the many contaminating proteins present in serum, several laboratories have succeeded in obtaining the pure protein using an extensive array of chromatographic and electrophoretic procedures. However, using displacement chromatography, Torres and his group were able to obtain 0.5 mg of the highly purified protein from 6 ml of serum using only three chromatographic steps. Torres said that for many applications it is advantageous to use HPLC columns in order to decrease the separation time and to increase the resolution. Therefore, Torres and his group have explored the use of CM-D's as displacers on HPLC anion-exchange columns. As an example, he showed the separation of the A and B genetic variants of the beta-lactoglobulins, which differ only by 0.1 pH unit in their isoelectric points. In order to demonstrate the high capacity obtainable by displacement chromatography, small 150-μl guard columns, which could be readily saturated with protein sample, were used. A single narrow-range spacing displacer was applied after the sample to displace β-lactoglobulin B from the column. A high affinity final displacer was then applied to displace β-lactoglobulin A. Up to 13 mg of these very similar proteins were totally separated by this method. Recently, Torres and his group have obtained an excellent first-step displacement separation with a column at 80-percent saturation in the purification of *E. coli* alkaline phosphatase, a more complex sample than that described above.

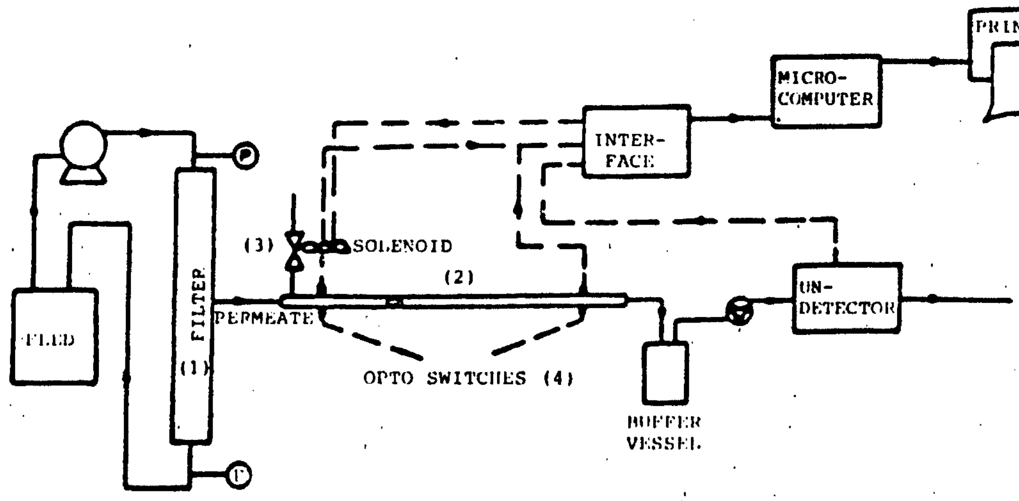

Figure 3. Experimental set-up with flowmeter and UV-detector inserted to allow continuous monitoring of flux and permeate protein concentration.

Torres and his group are presently attempting to achieve very-high-capacity enrichment fractionations of proteins present in very low concentrations by increasing the sample load and using small amounts of unfractionated displacers. Torres also mentioned that although the production of CM-D's is not overly complex, he and his coworkers are simplifying the process so that CM-D's can become more readily available. Torres thinks that displacment chromatography offers much higher capacities with improved resolution over standard elution methods if proper displacers are used.

4 ANALYTICAL TECHNIQUES AND PROCESS CONTROL

On-Line Monitoring

The on-line monitoring of flux and rejection during microfiltration of protein solution was reported by P. Heinemann (School of Chemical Engineering, University of Bath, UK). Heinemann said that although filtration techniques have a history of more than 40 years and are well established in both industrial applications and research laboratories, the recording of the performance data is still done in an old-fashioned way. The transmembrane flow rate is usually measured with a stopwatch and a calibrated volumetric vessel. This method does not allow data to be taken in very short intervals, which is of particular importance during the dynamic phase at the beginning of filtration, according to Heinemann. A further disadvantage is the requirement of continuous supervision if long-term measurements are needed. Protein rejection has already been monitored, according to Heinemann, with an on-line UV device but more commonly, off-line sample analysis is used. Therefore, Heinemann and her group developed a microprocessor-coupled system which allowed them to monitor transmembrane flow rate and protein rejection at the same time. This new technical approach offers the possibility of detecting changes in flux and rejection almost instantaneously. Heinemann and her coworkers have used the device to investigate the effect of pH and ionic strength on fouling of membranes in both the long and the short term. Figure 3 shows the experimental set-up with flow meter and UV-detector inserted to allow continuous monitoring of flux and permeate protein concentration.

Biosensors in Downstream Processing

The topic of biosensors for use in downstream processing was discussed by C.F. Mandenius (Department of Pure and Applied Biochemistry, University of Lund, Sweden). Mandenius said that although the biosensor has now been in use for more than 20 years, biosensors have mainly been analytical tools for analytical biochemistry. He added that due to their sophistication biosensors have not been available for routine analysis or for industrial applications. Except for a few cases, biosensors have not progressed from the research laboratories to the instrument manufacturers. Mandenius thinks that this is probably, though only to a minor extent, caused by the utility of the biosensors; it is to a greater extent due to the inertia in developing and introducing new apparatus and methods.

A variety of biosensors have been described so far. To classify them into categories of electrochemical, optical, and thermal biosensors is only one of several possible classifications. Depending on the definition of the concept that is chosen, other categories could be included; for example, sensors for measurement in biological fluids such as mass spectrometers, electrodes, semiconductors, etc., can also be included, according to Mandenius. He also said that with very few exceptions biosensors have not yet been employed in downstream processing. This does not mean, however, that biosensors are inappropriate choices for monitoring in this situation. On the contrary, Mandenius thinks that the already elaborated methods and principles of many biosensors coul , to a relatively large extent, be adapted to downstream process monitoring.

Mandenius thinks that the on-line concept is of particular value. Biosensors based on an immobilized enzyme reactor followed by a detection device--an electrode cell or a spectrophotometer flow cuvette--constitute versatile applications of this concept. For example, an enzyme thermistor with glucose oxidase developed by Mandenius and coworkers has been used to monitor the outlet glucose concentration of a lactase column for conversion of whey, and furthermore, used to control the addition of whey to the column reactor in order to optimize its yield. A related study employing a twin enzyme thermistor for simultaneous monitoring of glucose and sucrose was carried out by Mandenius and his group to control the outlet of an immobilized invertase column for sucrose inversion.

Enzyme thermistors have also been used to monitor chromatographic separation procedures. By mixing an enzyme with its substrate in the thermistor the relative activity, and thereby the protein concentration, is determined. By connecting the enzyme thermistor to the outlets of columns for gel filtration, ion-exchange chromatography and affinity chromatography, the enzymes hexokinase, lactate dehydrogenase, and glucose 6-phosphate dehydrogenase were detected after separation in studies carried out by Mandenius and coworkers.

Mandenius also stated that modern biotechnological processes require improved equipment for process monitoring. Production of penicillin and other antibiotics as well as insulin production with recombinant organisms still relies on traditional analytical methods. He thinks that enzyme thermistors can provide a specific, continuous analysis for several of these products using, for example, penicillinase, cephalosporinase, or insulin antibodies to analyze penicillin G. Such studies have already been carried out by Mandenius and coworkers.

Sampling in crude solutions often becomes a limitation to the application of analytical devices in process monitoring, according to Mandenius. Especially in biotechnology, these problems have been approached by connecting the sensor to an auxiliary device for sample treatment. Using a continuous dialysis probe, the analyte can be transferred to a pure buffer stream and subsequently to the sensor, as has been done by Mandenius and coworkers. On-line monitoring of blood and plasma serum has also been successfully performed by a "glucose monitor" with the dialyzer incorporated in the apparatus.

Another type of sensor is the membrane-gas sensor, sometimes referred to as a biosensor, which utilizes a gas-permeable membrane for the transfer and purification of, for example, ethanol and butanol, as carried out by Mandenius and coworkers.

Mandenius thinks that although the number of applications of biosensors to downstream processing is still somewhat limited, the use of biosensors in this field seems promising with respect to the amount and evidence of experience in closely related fields.

Downstream Processing Monitoring

Some approaches to downstream processing monitoring and the development of new separation techniques were addressed by T.A. Collinge (Microbial Technology Laboratory, Center for Applied Microbiology and Research, Porton Down, Salisbury, UK). Collinge said that until quite recently, developments in downstream processing equipment have received surprisingly little attention. Although effort has been consistently devoted to improving existing separation techniques, very few new techniques have been developed. Many downstream processes operate on a batch basis, whereas, the introduction of more flow line methods would particularly improve the potential for the automation of downstream processes. However, particularly lacking, according to Collinge, are devices capable of monitoring and controlling the range of techniques used in the separation and purification of biological products.

According to Collinge, cell harvesting continues to be largely carried out by semicontinuous flow centrifugation methods, although cross-flow filtration has received increased attention recently. A variety of cell rupture procedures are employed, but, although some techniques can operate in flow streams, they are not routinely incorporated in-line. Liquid chromatography continues to occupy a central position in protein and enzyme purification and there have been a wide range of developments in chromatography media. More recently, the development of more rapid HPLC, FPLC, and immunoaffinity methods have become popular for the final stages of protein purification, particularly for recombinant therapeutic products.

The progress of cell harvesting and cell breakage are not routinely monitored, according to Collinge, although off-line microscopic examinations or spectrophotometric assays are used. The purification of proteins and other materials is usually analyzed off-line. The only on-line monitors widely available are UV absorption measurements of protein concentration. Conductimetric and pH monitoring of industrial-scale liquid chromatography eluants (for example, for gradient elutions) has only been recently introduced. Therefore, Collinge essentially presented a review of some recent developments in new separation methods and improvements in existing methods, together with some new approaches to on-line monitoring.

Productivity Monitoring. The majority of microbial enzymes or protein products are maintained within the cell, either within the cytosol or periplasmic space. Relatively few are secreted into the growth medium, although with the advent of molecular genetics techniques, it has become possible to engineer secretion of a range of protein products either into the periplasmic space or into the growth medium itself. Thus, in the case of secreted or periplasmic products, it is possible to conceive of monitoring protein production without disrupting cells, according to Collinge. However, he added that in many cases it is still necessary to consider rapid and automatable methods of disrupting or permeabilizing small volumes of cells to monitor the production of proteins and other species in the cytosol.

Collinge stated that there are a number of methods capable of assessing the cell interior for assay purposes (for example, solvent permeabilization of cell membranes, ultrasound disruption, enzymic disruption of the cell envelope), but most of these methods completely disrupt or permanently damage the cells. However, according to Collinge, when cell suspensions are treated with high-voltage pulses. it is possible to cause pores to form in the cell membrane of sufficient size to introduce macromolecules (for example, DNA) through the cell membrane of bacteria and eukaryotic cells. Depending largely on the amplitude of such high-voltage pulses, it is possible to introduce either permanent or resealable pores. Consequently, according to Collinge, it is possible to conceive of monitoring enzyme and other activities in whole cells without disrupting the cells and, depending on the toxicity of any reagents used, without damaging the cells.

Collinge and coworkers are particularly interested in monitoring the fermentation and purification of *Erwina* asparaginase used in the treatment of acute lymphoblastic leukemia, particularly in children. Although in some strains, the enzyme is associated with the periplasm, in others, it is intracellular. Colorimetric assays measure the production of ammonia from asparagine, which proves difficult in the presence of high background levels of ammonia (for example, in culture media) and because of airborne ammonia contamination. *Erwina* can survive electroporation treatment, according to Collinge, but cells are killed under certain electroporation conditions. The asparaginase-catalyzed reaction can also be monitored conductimetrically, particularly appropriate because the presence of whole cells does not unduly interfere with such assays. Although naked electrode techniques are normally used for conductimetry, Collinge and his group have recently developed a noninvasive oscillometric technique where field-induced coupling through the measurement cell wall is used to monitor conductivity to high resolution. The measurement cell is configured within the feedback loop of a wideband amplifier, such that the oscillometer outputs changes in frequency for corresponding changes in conductivity. In order to minimize the effect of temperature drifts, a dual cell configuration was used. Although this technique has been configured both as a test-tube holding and dip probe designs, in this case, Collinge and coworkers employed glass-walled flow line cells suitable for in-line application. Using their procedure these researchers were able to assay the production of asparaginase on mixing of asparagine with culture sample throughout the course of batch growth.

Cell Harvesting. Collinge said that acoustic resonance densitometry can be used to monitor the production of biomass in production scale cultures. Such techniques are now being developed as both in-line and dip probe configurations. The linear range of such biomass measurements was demonstrated to extend to equivalent optical densities of approximately 500. Thus, it is capable of monitoring the concentration of harvested cell slurries (approximately 15 percent weight/volume) as well as the biomass of growing cultures and would appear particularly appropriate for the in-line monitoring of continuous-flow harvesting of biomass and cell debris.

According to Collinge, the high running costs and potential hazards of centrifugation (energy costs, rotor replacement, and aerosol production) have led to the consideration of membrane filtration techniques, particularly tangential or cross-flow filtration. Collinge thinks that the main reason that this technique has not found more wi .pread application in production process is because membranes can rapidly become fouled, causing a progressive fall in harvesting throughput. This can be avoided to some extent by ensuring that sufficiently high shear forces exist at the surface of the membrane, which are usually controlled by altering the rate of flow. Acoustic resonance densitometry can be used to monitor and control the operation of such systems. Membrane fouling can be further minimized, according to Collinge, by using vibrating membrane techniques, where membrane movement can be driven piezoelectrically or electromagnetically. Prefilters can also be used to minimize microbial fouling. Collinge and his group have recently demonstrated that over 9C percent of culture biomass can be harvested noninvasively using ultrasound fields, which may well be useful as a nonmembrane-requiring "prefilter" technique and would appear particularly useful for harvesting potentially dangerous pathogenic organisms in a fully contained manner.

Collinge said that the examination of magneto-mobile and ferro-organisms has stimulated some interest in the harvesting of organisms by strong magnetic fields. Organisms suspended in media containing iron II ions can be deflected in a flow stream passing close to the pole face of a superconducting magnet. In the presence of such fields, particles may experience attractive or repulsive forces based on one or more of three magnetic properties: ferromagnetism (strongly magnetic), paramagnetic (weakly magnetic, positive susceptibility) or diamagnetic (nonmagnetic, negative suceptibility). Thus, according to Collinge, it is possible to conceive of more selective separations depending on either the natural or contrived (for example, addition of ions) magnetic properties of the particle. Such techniques would have the advantage that they are nonenergy requiring. Superconducting magnets providing fields of a few Telsa are relatively expensive, but cheaper superconducting magnets are being developed, according to Collinge.

Cell Disruption. Collinge said that the extent of cell rupture is not routinely monitored and any tests carried out usually involve either microcopic examination or assay of the release of intracellular biochemical species (for example, enzymes). According to Collinge, laser particle sizing techniques can be used to monitor the extent of cell breakage. Also, photon correlation spectroscopy (PCS) and other light scattering techniques can now be applied, using fiber optics and optelectronics, with relatively low-cost signal processing, rendering them highly applicable to bioprocess monitoring.

Monitoring of Protein Purification. PCS can also be used to monitor the molecular size of proteins eluting from a chromatography column. Although PCS relies on the measurement of diffusion coefficients, in the case of proteins, it can be applied in flowing streams from both liquid chromatography and HPLC columns and can sample rapidly enough (about 10 seconds) to be considered as an in-line continuous-monitoring technique, according to Collinge. There is reasonable agreement between the measured diffusion coefficient of the majority of proteins and their reported molecular weights. Low-angle laser light scattering measurements can also be used to measure molecular weight.

The separation of proteins of different molecular weights has been monitored by the application of photon correlation. In relatively crude samples the monitoring of polydispersity provides an estimate of the purity of the preparation, according to Collinge. At low protein concentrations, UV absorbance monitoring of protein concentration is more sensitive than monitoring the intensity of laser light scattered. However, the upper range of scattering intensity protein concentration measurements is much higher than UV absorbance monitoring, whose range is often exceeded in production-scale purification procedures. Measurements of the intensity of laser light scattered at 90° can also provide a direct estimate of molecular weight, except in the case of large particles, where low-angle scattering measurements are required, according to Collinge. Thus hydrodynamic diameter (from diffusion coefficent measurement) and molecular weight data can be obtained simultaneously from laser light scattering measurements.

Collinge said that there have been relatively few developments in biosensors capable of monitoring the levels of specific proteins, and most research has concentrated on the use of affinity binding reactions at solid/liquid interfaces. However, liquid/liquid interfaces have also been examined. Dyes used for the preparation of protein affinity matrices have been polarized at liquid/liquid interfaces by attaching a hydrophobic chain to the affinity dye. In view of the very wide range of protein products, it would appear commercially difficult, according to Collinge, to develop biosensors for any specificity which would be expected to have a sufficiently large market. One approach could be the provision of biosensor kits where the user could alter the specificity of the device. An alternative approach would be to produce an array of biosensors where the individual responses of each sensor to particular proteins could be mathematically analyzed to provide sufficiently specific monitoring for a wider range of applications.

5 LIQUID-LIQUID EXTRACTION

The topic of liquid membrane extraction was discussed by D.L. Pyle (Department of Food Science and Technology, Food Studies Building, University of Reading, UK). Pyle considered the potential of liquid membrane technology as a means of separating biotechnological products. He also reviewed work in this field and presented his group's work on the separation of carboxylic acids from real and simulated broths.

The basic principles of a liquid membrane are shown in Figure 4. By virtue of its immiscibility the so-called "membrane" phase separates two other liquid phases. In most applications, according to Pyle, the membrane phase is an organic solvent, separating two aqueous phases. Solute is transferred from phase 1 into the membrane-phase 2 and then into phase 3, where conditions prevent back-extraction of the solute: for example, it may contain a reagent which is insoluble in the membrane. A number of modifications to this basic scheme may be advantageous in various ways. Two methods of achieving the membrane system are shown in Figure 4. In the liquid membrane emulsion (LME) the internal phase (phase 3) is emulsified at high shear in the membrane phase, which forms a dispersion of droplets (typically 1-10 μm). A surfactant is normally required to stabilize the emulsion. The emulsion is then itself dispersed as globules 2 to 5 mm in diameter in the continuous external phase in a mixing vessel (with slow stirring) or in a column contactor. The "membrane" is thus the continuous phase within the droplets. A supported liquid membrane (SLM) can be achieved by impregnating a porous solid film with the membrane phase

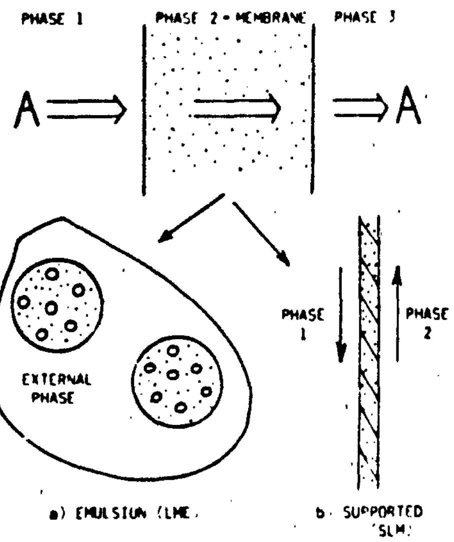

Figure 4. Liquid membranes.

and using this device to separate phases 1 and 3. The solid support is typically 25 to 100 μm thick with a porosity of 70 percent and a mean pore size of around 0.02 to 1 μm.

Pyle said that the principles of separation by LME and SLM are the same, but each appears to have some potential advantages and disadvantages. LME systems possess the advantage of very large specific interfacial areas; they depend on the careful choice of system, including surfactant; they can be susceptible to swelling and breakage under process conditions. Following extraction, emulsion breakage and phase separation are required. Particular surfactants may produce significant transfer resistance at the interfaces as well as suppressing circulation of the internal phase and the emulsified droplets. Both these effects will tend to reduce internal transfer rates. The use of surfactants and organic membrane phases may present problems of compatibility in some biological systems.

Pyle said that there has been less study of SLM systems, but retention of the membrane phase on the support is known to present problems. Relatively large interfacial areas could be achieved by using hollow fiber supports, but support blockage and concentration polarization could also lead to problems. However, according to Pyle, both systems offer the considerable potential advantages of extraction and stripping in one process operation, and of concentration--that is, transport against the concentration gradient--and that continuous operation appears to be feasible.

Applications in Biotechnology. Pyle stated that most interest in the potential advantages of liquid membrane technology (high specific transfer rates, the ability to separate and concentrate from dilute solutions, compactness, etc.) has been in relation to metals extraction. In contrast, there are few reports of direct relevance to biotechnology, and most of them relate to laboratory-scale studies. Early studies on acetic acid separation involved the diffusional extraction, without facilitation, of unionized acid; back diffusion was prevented because the acetate ion formed by reaction with sodium hydroxide in the internal phase is insoluble in the organic membrane. In recent work, Pyle and his group used a tertiary amine as a facilitating carrier. LME's have been tried for the separation of biochemicals with ionizable functional groups such as amino acids, phospholipids, etc. by Pyle and his group as well as by other investigators. Using concentrations of up to 15 g/l of L-phenylalanine it was possible to separate and concentrate the phenylalanine anion with chloride as the counter ion. However, Pyle emphasized that the selectivity of most such systems is still extremely low and represents a great challenge.

Pyle mentioned that there have been studies of several LME/enzyme systems, in which the enzyme is immobilized in the internal phase. Some of these studies have included the use of cell homogenate and, subsequently, whole living cells of *M. dentrificans*, in which the nitrogen reductase system catalyzed the reduction of nitrate and nitrate ions. K. Schügerl's group at the University of Hannover, West Germany, have used enzymatic systems as the basis for the production of L-amino acids, the separation of penicillin G, and other biochemical systems. Pyle emphasized that while these various studies demonstrate the potential of LM systems in biotechnology, they also spotlight a variety of problems. Among these are: emulsion formulation to achieve compatability and stability; emulsion swelling; the development of highly selective systems; the lack of trials on real rather then "model" systems; recovery from the emulsion phase. Pyle said that he was unaware of any studies of supported membranes, but that many of these same problems (except emulsion stability and breakage for product recovery) are likely to occur.

Pyle mentioned the use of LM extraction *in situ* with a fermentation or enzymatic reaction. He considers this idea very attractive since continuous product removal could often relieve kinetic inhibition. The work of Pyle and his group on extracting citric and lactic acids was partly motivated by this aim. It appears that LME systems can be compatible with enzymatic and microbial processes. Preliminary studies also suggest that systems compatible with *A. niger* are possible. Other potential applications which might be explored include their use in affinity and other protein separations, the use of multiphase aqueous systems, and the employment of a wider range of enzyme systems. Pyle said that the choice and development of surfactants, solvents, and carriers clearly represent key targets.

Extraction of Organic Acids. Citric and lactic acids are both produced on a large scale by fermentation. Pyle, in reviewing the potential for solvent extraction noted that the classical recovery methods involve precipitation of the calcium salt, treatment with sulfuric acid, further purification and, finally, evaporative crystallization. Therefore, there is a good incentive, according to Pyle to seek alternative separation methods. *In situ* removal would be particularly promising to reduce the effects of product inhibition. Pyle and his group are currently studying both organic acid systems at Reading University and Imperial College. It appears that tertiary amines (trioctylamine as Alamine 336) perform well as extractants for both citric and lactic acids, using a predominantly aliphatic solvent (Shellsol A). While early work concentrated on the use of aqueous NaOH as the internal phase it was found that good extraction and emulsion stability properties could be achieved with sodium carbonate solutions. For this system, Span 80 with a membrane strengthening agent (Lubrizol 3702) was an effective surfactant.

Work on the batch extraction of citric acid from solutions of up to 10 percent w/w by Pyle and his group showed the following:

- Emulsion stability (indicated by a reversal of the trend in external concentration versus time) is significantly affected by the concentration of surfactant and strengthener and by emulsification time. Stability can be maintained in excess of 20 minutes with 3-percent v/v and 2-percent v/v at 5 minutes emulsification or with 5-percent and zero-percent Span 80 and Lubrizol, respectively, after 10 minutes emulsification.
- Carrier concentrations of 5-percent v/v Alamine give good stability and extraction kinetics.
- Sodium carbonate concentrations of up to 20-percent v/v give excellent results.
- Extraction efficiency improves with decreasing external phase pH, showing that undissociated acid is taken up and suggesting a transfer mechanism.

In studies of lactic acids, Pyle showed the time course of a typical batch extraction of lactic acid, as a 25.2 g/l solution. Emulsification was carried out in an ice-bath to encourage stability. It was seen that the transfer rate of lactic acid was extremely high: around 98-percent extraction could be achieved within 4 minutes. The emulsion system used (which can be improved upon) was slightly unstable, as evidenced by the reversal in the direction of net transfer. Moreover, neglecting any emulsion swelling, the internal phase conditions after 4 minutes of extraction corresponded to a sodium lactate concentration of 186 g/l, representing an approximately sevenfold increase in product formation from the starting material.

Pyle concluded that liquid membrane processes offer significant and unexplored potential for application to biological systems. There are, however, many experimental and theoretical problems still to be resolved before fully developed processes are likely to be available and before a definitive critical assessment of the technology's true potential can be made.

Reactive Extraction in Biotechnology

The subject of reactive extraction in biotechnology was discussed by K. Schügerl (Institute for Technical Chemistry, University of Hannover, West Germany). Schügerl said that biotechnological production processes are characterized by diluted aqueous solutions in which the valuable product is accompanied generally by several chemically similar compounds. The first stage of the recovery of these products usually is enrichment. It is followed by several purification steps. The extraction can be one of these recovery or purification processes. The solvent extraction of primary metabolites is hampered by their low partition or distribution coefficients and high solubility of the organic solvents (hydrocarbon and hydrocarbon derivates) in the aqueous phase. The solvent extraction of secondary metabolites at the optimal extraction pH-value frequently becomes problematic because of their pH-instability. Sometimes the metabolites are insoluble in apolar organic solvents which have low solubility in the aqueous phase. Schügerl said that this problem can often be solved by the use of solvents which have stronger and more specific solvation bonds than the carbon-bonded oxygen-bearing extractants or those which form ionic bonds. Such extractants are organophosphorus extractants and high molecular weight aliphatic amines, which form ion pair complexes with the solutes. Schügerl discussed the use of these two types of extractants in biotechnology. Since either strong and specific solvation bonds with definite stoichiometry or ionic interaction exists between solvent and solute, these extraction processes were called reactive extraction by Schügerl.

Reactive Extraction of Primary Metabolites. Most primary metabolites are alcohols and carboxylic acids. According to Schügerl, to date no extractants are known which form solvation bonds with alcohols which are strong, selective, and reversible enough to be used in reactive extraction. Therefore, Schügerl considered only acids in his talk. The two most important metabolites are acetic and citric acids.

Phosphorus-bonded oxygen-donor extractants contain a phosphoryl group, which is a strong Lewis base. Furthermore, these extractants coextract low amounts of water, and their solubility in water is low. The distribution coefficient K_D, which is the concentration ratio of the sum of the solute components in the organic phase to that in the aqueous phase, is generally used for the characterization of the distribution of the solute between the two phases in the case of reactive extraction. In Table 5, K_D values of acetic acid with tributylphosphate (TBP) and trioctyl phosphine oxide (TOPO) in different diluents at high dilution are compared. In contrast to the commonly used organic solvents (carbon-bonded oxygen-donor extractants), in which the distribution coefficients are generally lower and their solubility in the aqueous phase is higher, the extractant-diluent systems shown in this table have K_D values higher than one, and their solubility in water is by an order of magnitude lower. The diluents and the acid concentration have considerable effect on the distribution coefficient. The extraction power increases as the number of direct C-P linkages increases. Temperature has also been found to influence the distribution coefficient of citric acid. With increasing temperature, the distribution coefficient is reduced considerably. This behavior is used for the recovery of citric acid from the organic phase.

Table 5
K_D values of acetic acid with TBP and TOPO in different diluents at high dilution

Extractant	Dilutent	K_D
TBP	hydrocarbon	2.3
TOPO	2-ethyl hexanol	1.12
TOPO	2-heptanone	2.83
TOPO	Chevron 25	2.01

According to Schügerl, when using high molecular weight aliphatic amine extractants, a proton transfer occurs during the extraction, and the acid prevails in the organic phase as an amine-acid ion pair. In some cases, the amine base can take up acid in excess. The extraction of acetic acid with different high molecular weight primary, secondary and tertiary amines has been studied. It appears that primary amines have a high mutual solubility with water, secondary amines yield high values of distribution coefficients. Actually, they provide the highest distribution coefficients (for example, K_D=160 with Adogen 283 D in 2-ethyl-1-hexanol). However, they are subject to amide formation during regeneration of the extractant by distillation. Tertiary amines are very effective, but they generally result in lower K_D values than secondary amines, according to Schügerl.

Table 6 shows the distribution coefficients of citric acid in water (Alamine 336-diluent system at different citric acid concentrations). The diluents and the acid concentration have considerable influence on the distribution coefficients. As the extractants are fairly expensive, it is important that their loss, due to their solubility in the aqueous phase, is low, according to Schügerl. Long-chain tertiary amines are practically insoluble in the aqueous phase at acid concentrations below 10 weight percent. For example, in a 1-weight-percent acid solution, the solubility of Alamine 336 is less than 10

Table 6
Effect of diluent on the extraction of citric acid with Alamine 336 at 25°C

Diluent	Conc. of citric acid in raffinate phase 10^3 (M)	K_D
n-hexane	14.30	2.9
	382.00	1.9
toluene	4.93	10.8
	358.00	2.1
111-trichlorethane	3.37	16.4
	348.00	2.2
ethylacetate	2.45	23.0
	275.00	3.1
MIBK	2.07	27.5
	276.00	3.1
cyclohexanone	1.87	30.5
	220.00	4.2
2-ethylehexanol	0.83	70.0
	351.00	2.1
iso-amylalcohol	0.67	87.8
	305.00	2.7
dichlormethane	0.63	92.3
	372.00	2.0
nitrobenzene	0.60	97.6
	340.00	2.2
n-butanol	0.35	168.3
	274.00	3.1

parts per million. Recently, encouraging results have been achieved by the combination of different types of extractants. For example, dioctyl phosphate was combined with trioctyl amine.

Reactive Extraction of Secondary Metabolites. In the biotechnological production of several antibiotics and vitamins, solvent extraction is used as the first recovery step from the fermentation broth (Table 7). Some of them are weak acids. To extract them by an organic solvent, it is necessary to reduce the pH-value during the extraction below the pK value. Some of them are very unstable at these low pH values. A typical representative of such antibiotics is Penicillin G. This compound (pK=2.75) can only be extracted from the fermentation broth by organic solvents (for example, butylacetate). The industrial process, according to Schügerl, is performed at pH 2 at 0° to 2°C in centrifugal extractors. However, he said that because of the compound's instability at this pH-value, the recovery losses due to its decomposition are considerable. The reactive extraction of Penicillin G with secondary or tertiary amine extractants in conventional solvents (for example, butylacetate) as diluents can be performed at pH 5, at which Penicillin G is stable. The re-extraction can be carried out at pH 7 with a high degree of extraction as has been done by Schügerl's group. These researchers also worked out the thermodynamics and kinetics of the extraction, and the process was performed in bench-scale and pilot-plant-scale extractors with fermentation broth. The process was simulated by means of a mathematical model and applied to the pilot plant. The scale-up of the process is easy to perform, according to Schügerl. The reactive extraction of Penicillin G was also carried out successfully in three different types of extraction columns, in

Table 7
Liquid/liquid and solid/solid extraction of some biologically produced antibiotics and vitamins

Product	Medium	Solvent	pH
Macrolide	zF	MIBK, EtAc	s
Novobiocin	zF, NA	AmAc, Alcohol	6
Oxytetracycline	NA	n-BuOH	
Penicillin G	zF	n-BuAc, AmAc	2
Polyene antibiotics	zF, M	n-BuOH	
Riboflavin	M	H_2O-steam	
Tetracycline	zF	n-BuOH, MIBK	8.5
Tylosin	zF, NA	EtAc, AmAc, $CHCL_3$	
Virginiamycin	zF	MIBK	s
β-Carotene	M	Petrolether	

zF = from cell-free medium
M = from mycelium
NA = after adsorption
s = acidic
a = alkaline

a mixer settler, and in different centrifugal extractors. The recovery losses of Penicillin G were lower than 1 percent, and the amine extractant losses were negligible.

Schügerl concluded his talk with the following statement. By means of reactive extraction, especially by means of ion pair extraction, the performance of several extraction recovery processes in biotechnology can be improved considerably. He also stated that the advantages of reactive extraction are: (1) the loading capacity of the solvent can be increased considerably; (2) the selectivity of the recovery can be improved; and (3) the recovery can be performed under conditions in which the recovery losses due to the decomposition of the product are low.

Extraction in Aqueous Two-Phase Systems

The topic of extraction in aqueous two-phase systems for biotechnology was addressed by B. Mattiasson (Department of Biotechnology, Chemical Center, University of Lund, Sweden). Mattiasson said that extraction is a unit operation that gives several advantages, making it attractive in separation processes. It is relatively easy to scale up, it does not normally involve complicated and expensive equipment, and there is a vast experience in chemical industry on various applications of this separation technology. When selecting an extraction medium one has to take into account that the substances to be separated show differences in solubility in the two media, and that they are not denatured by the extraction procedure. When dealing with biological molecules these two parameters, solubility and stability, are hard to match with the extraction media normally applied in industry. Several of the criteria used when selecting a proper extractant for biotechnological applications are listed in Table 8. Mattiasson said that when operating with biological macromolecules or cells it appears that a rather limited number of suitable solvents are available. The introduction of aqueous two-phase systems has broadened this list substantially.

Table 8
Criteria for selection of conventional extraction media

* Unilateral partition coefficient for the product
* Not toxic to the biological system
* Easy separation of product from extractant
* Cheap and available in large quantities
* Possible to sterilize
* Immiscibility with the water solution
* Inability to form stable emulsions with the biological material
* Non-flammable
* Not toxic to personnel

According to Mattiasson, biological macromolecules, cell particles, and cells partition in two-phase systems. The partition behavior is a reflection of the surface properties of the material to be partitioned. The driving force for partition has been divided into several separate factors denoting the contribution to the overall partition by electrical charges, hydrophobicity, biospecificity, size, and conformation. According to Mattiasson, each one of these factors may be made to play a dominating role in a partition experiment. Charges, hydrophobicity, and size are three parameters that may very well be determined for a biochemical entity and thus may be used to predict the partition behavior. So far, however, there are no basic theories for the description of partition behavior, which is why, according to Mattiasson, most predictions are still based on experience rather than on real understanding of the underlying mechanisms. Some recent papers have addressed the question of understanding the basic principles of the formation of aqueous two-phase systems (King et al., 1986; Gustavsson et al., 1985). It is well known that the partition behavior of a molecular entity is much easier to predict for large molecules than for small. Thus, as seen in Table 9, taken from work by Mattiasson and his group, cells and cell organelles have a tendency to partition with extreme patterns, whereas proteins and even smaller molecules are more

Table 9
Partition constants for various biochemical entities

Entity	Partitioning
Small molecules (substrates/products)	0.1 - 10
Proteins	0.01 - 100
Particles (cells, gel particles, etc.)	extreme partitioning
Separator molecules	0.001 - 1000
Separator particles	extreme partitioning

unpredictable. A technique to improve the partition constant is to introduce affinity binding to a ligand with a predetermined partition behavior. According to Mattiasson, an improved partition behavior is achieved by this complex formation. The aqueous two ase systems do not tulfill all the requirements for an ideal extractant. For example, a more extreme partitioning behavior would be desirable.

Aqueous two-phase systems have a high water content and a low interfacial surface tension and are regarded as being biocompatible. The polymers used have been shown to be nontoxic even to mammalian cells in partition experiments. Polyethylene glycol (PEG) has been used in other cases for cell fusion, and one has to be careful regarding membrane effects on labile cells, according to Mattiasson. Concerning microorganisms, no negative effects have been observed. In many cases, it has been reported that polyols, like the polymers used in the phase systems, stabilize enzymes. This is also the case in an enzyme-reaction run in an aqueous two-phase system. Also, product can easily be separated from the extractant. It is important that the substance partitioned to one of the phases can be easily separated from the phase components. Three different cases are the separation of particles, of macromolecules, and of small molecules. In many phase systems most macromolecules favor the bottom phase, so in order to get some purification the compound to be re-extracted is forced to the top phase. In the following step this compound may be recovered and extracted to a bottom phase of, for example, salt under altered conditions. Finally, membrane filtration separates the salt from the compound. Alternatively, ion-exchange or affinity chromatography may be applied. Particulate matter may be separated off using filtration or sedimentation from the favored phase or the interface. When using separator particles in the phase system the particles are filtered off and placed in a chromatographic column before washing and elution of the bound material (Mattiasson and Ling, 1985).

Low molecular weight compounds have a tendency to partition rather equally between the phases, according to Mattiasson. Extraction of small molecules is of interest in, for example, bioconversion. The recovery of the small molecule from the polymer solution may be achieved by membrane filtration or by applying specific sorbents, as has been done by Mattiasson and his group.

A broad spectrum of polymers has been tried for producing aqueous two-phase systems, according to Mattiasson. Most such systems are acceptable for the research situation, but when coming to scaling up, quite different criteria must be met. The cost of the polymer now becomes very important. Also, the time for phase separation, the amount needed for formation of an aqueous two-phase system, and the possibility of recycling the polymer must be considered. PEG is considered a suitable polymer for the top phase and dextran has been used for many years as the bottom phase component. However, dextran is much too expensive for scaling up. A starch-based polymer, Reppal PES, has been developed that meets the price requirements and in most respects behaves as the dextrans (Nilsson et al., 1987). Reppal PES is modified with hydroxypropyl groups to an extent that it retains the property of biodegradation. By increasing the degree of substitution of the Reppal PES, a nondegradable polymer is produced. Mattiasson said that for large-scale applications the biodegradation should be of interest to avoid unnecessary costs. In many cases, it has proven efficient to use salt as the bottom phase component. PEG/potassium-sulphate systems have been used for the isolation of enzymes on a large scale. Mattiasson said that according to an economic analysis of the primary separation steps for cell debris separation, the cost of an extraction step with aqueous two-phase systems was estimated to be three-quarters of the price of the phase components (PEG and salt). The total cost was at best equal to the cost of tangential flow microfiltration. This analysis showed that the price of the polymers is nearly the only parameter that determines the economic feasibility of the method of extraction. In another evaluation the cost of extraction was only half or two-thirds the cost for other primary steps used for recovery of intracellular enzymes.

In an aqeous two-phase system, both phases consist of water to a large extent (85 to 90 percent), yet they are immiscible and form phases within a few minutes after mixing. Frequently used compositions of such phase systems are PEG/polysaccharide and PEG/salt. The polysaccharide may be dextran or hydrolyzed starch. Examples of salts include sodium phosphate and other salts having a multivalent anion. Aqueous two-phase systems are obtained by mixing two water solutions of phase components above their threshold concentrations. Their suitability for the separation of biological material is reflected by the low interfacial tension, which may be a fraction of a dyne/cm, while conventional phase systems (composed of water and an organic phase) may have a 100-fold higher tension, according to Mattiasson. The high water content and the low interfacial tension have been regarded to be two reasons for the good biocompatibility observed in such phase systems.

Applications of Aqueous Two-Phase Systems in Biotechnology. Mattiasson said that there are reports on several different areas of application within the biotechnological area. The first reports on aqueous two-phase systems dealt with the separation of biomolecules in small scale in the laboratory. At that time, proteins were often used as tracers to follow how the partition behavior changed when the conditions in the phases changed. Later the results from these studies were applied in larger scale partitioning of proteins with the aim of isolating proteins. At a later stage the resolution was improved further by introducing affinity-mediated partitioning. This technique may also be used for purification of proteins, according to Mattiasson. Affinity binding involved biospecific recognition and formation of a specific complex. Since the same steps are used when setting up binding assays it was quite natural to try this technique, and partition affinity ligand assay (PALA) was thus established. Finally, the efficient mixing and the good mass transfer, together with the knowledge that a totally assymetric partition pattern could be quite easily achieved when operating with cells led to investigations of the potentials of aqueous two-phase systems for extractive bioconversions. In these systems the biocatalyst could be regarded as being immobilized in the droplet as long as stirring continued, according to Mattiasson. If left unstirred, phase separation took place very rapidly. In an effort to "freeze" the situation in the emulsified state, the droplets of the bottom phase were solidified, and thereby a simple and gentle bead formation procedure was achieved (see Table 10).

Purification of Proteins by Partition in Aqueous Two-Phase Systems. The basic principles for the partition of a protein in an aqueous two-phase system has been covered in many papers. However, according to Mattiasson, the whole area is so far based on experience; no solid theoretical background has yet been presented. The partition behavior is said to reflect the surface properties of the protein to be partitioned. The partition behavior can be influenced by changing various parameters--such as polymer concentration, molecular weight of polymers used, chemical character of the polymer, or the ion environment--and thereby the

Table 10
PALA--systems studied

Antigen	Separator	Other Reactant	Label	Concentration Range	References
Glucose	PEG-ConA	Glucose	Hrp	20-1000	Mattiasson and Ling, 1980
Staphylococci	(Staph)	IgG*	I	10^6-10^7	Mattiasson et al. 1981
Staphylococci	PEG-Staph	IgG*	I	10^5-10^7	Mattiasson et al. 1981
Streptococci	(Ab)*	Ab*	HRP	2500-1,000,000	Ling et al. 1982
Streptococci	PEG-Strep	Ab*	I	25,000-100,000	Ling et al. 1982
$\beta_2$microglob	PEG-Ab	β_2m*	I	(3-96 µg/l)	Ling and Mattiasson, 1982
$\beta_2$microglob	PEG-Staph	β_2m*/Ab	I	3-96 µg/l	Ling and Mattiasson, 1982
Digoxin	(Ab)	Ab/Digoxin*	I	1-8 nM	Mattiasson, 1980
T3	(Ab)	Ab/T3*	I	1-6 nM	Mattiasson and Eriksson, 1982

Explanations: Label: HRP = Horseradish peroxidase; I = Iodine-125
*denotes a labelled reactant

interfacial potential. When a protein is to be isolated from a complex mixture, it is important to find the right conditions. This is usually done via many small-scale experiments in a rather time-consuming process, according to Mattiasson. When the right conditions are found it is fairly easy to scale up. When designing a partition experiment it is important to optimize yield and partition behavior versus the rest of the components in the sample--i.e., the purification factor. It has been found that an enrichment of the protein by a factor of 10 is achieved in most cases. The great advantage, however, is that cell debris, DNA, and other components that may disturb subsequent purification steps can often be removed without time-consuming centrifugations or membrane filtration steps. Thus, the strength of the spontaneous partition of proteins in aqueous two-phase systems lies in the fact that disturbing material is efficiently removed in one step, according to Mattiasson. Under favorable conditions, it is even possible to achieve a high purification in this first step. When the difference in partition factor is not large enough, a series of extraction steps may be applied. In a counter-current distribution (CCD) machine, such separations may be performed on an analytical level. If, however, a larger scale is to be applied then multibottom extraction columns can be applied. The resolution in such multistep partitions is very high, and it has been demonstrated in the separation of cells during differentiation.

Affinity Partitioning. When an affinity partioning takes place in free solution, a soluble complex is formed. The partition behavior of this complex is dependent upon the characteristics of the individual components. In such an ideal situation the ligand favors one phase to such an extent that the complex will also partition to the same phase. Affinity partitioning is based upon the principle that the complex should have a different partition pattern as compared to the free target molecule. When the ligand is not exhibiting extreme partition behavior it has to be chemically modified prior to use. In most cases, proteins partition to the bottom phase and the investigator's objective has therefore been to selectively transfer the largest molecule to the top phase. To make the ligand favor the top phase, chemical modifications with the top phase polymer have been used. Since PEG is the major top phase polymer used, this has meant coupling of PEG to the ligand.

A large number of coupling reactions have been applied to PEG, and today there are several alternatives available (Harris, 1985). The affinity partitioning takes place as follows: The modified ligand is mixed with the homogenate and after proper binding has taken place the phase system is added. After proper mixing, phase separation takes place and the affinity complex is then found in the top phase. A normal procedure for isolating a pure target protein freed of ligand and phase components has involved the use of a second partition step when the top phase has been mixed with a fresh bottom phase under dissociating conditions. During mixing of the phases, dissociation of the complex takes place and the target protein will then partition according to the spontaneous pattern--i.e., it will be recovered from the bottom phase. Isolation of protein from phase components may be done using ion-exchange chromatography of membrane filtration. The ligand may be recovered from the top phase and reused.

Affinity partitioning as described above involves the need of modifying each individual inhibitor or ligand to go to the top phase, according to Mattiasson. However, this may be impractical and in some cases even unsuitable. Therefore, Mattiasson and his group developed the strategy of applying second separator molecules having an affinity for the ligand-target protein complex and a partition behavior strongly favoring the top phase. An example of this is the protein, avidin, that was PEG-modified, and the ligand was modified with biotin residues in a mild and gentle modification reaction. A similar approach was applied in immunoaffinity partitioning by applying protein A as the separator molecule to be modified for extreme partitioning. As stated above, even if soluble molecules may be partioned to one phase, it is much easier and more predictable to use particles. Therefore, second separator particles were developed as well.

<u>Extractive Bioconversions.</u> A general characteristic of biologically catalyzed processes is that they are equilibrium processes. This means that a high concentration of product will inhibit the rate of catalysis to generate more product. This is a very useful arrangement when in operation in the living cell, but when applying biological catalysts in processes, this causes severe limitations, according to Mattiasson. In many cases biotechnological processes have been characterized as being rather slow and producing streams in dilute product. In addition, in the case of product inhibition there may be other cases when it is desirable to efficiently remove a compound as, for example, when working with very sensitive compounds. A review on extractive bioconversions has recently been published by Mattiasson and Larsson (1985).

Application of aqueous two-phase systems offers certain advantages when designing an extractive bioconversion, according to Mattiasson. The biocompatibility of the phases is an important feature in this context. As stated above, it is quite easy to design systems where cells partition exclusively to one phase, whereas the pattern for enzymes is slightly more complicated and for small molecules may be unpredictable. Thus, the biocatalyst may very well be confined to one phase, and in the case of low molecular weight product, the product might be recovered from both phases. In such cases, Mattiasson thinks that it might be of interest to improve the partition pattern of the product to the phase where the biocatalyst is absent. This can be done by changing the concentration of the polymers or by adding other polymers. Still another reason to use extractive bioconversion was demonstrated in steroid bioconversions where under normal conditions the limited solubility of the substrate/product restricts the efficiency and yield of that process. In Table 11 some different systems applied to aqueous two-phase systems are listed; the phase systems used as well as certain features of the systems are given. Running the bioconversion in an aqueous two-phase

Table 11
Extractive bioconversions

Process studied	Catalyst	Phase system	Reference
Starch > glucose	Amyloglucosidase	PEG/crude dextran	Larsson and Mattiasson, 1984
Starch > glucose	Amyloglucosidase	PEG/starch	Larsson and Mattiasson, subm
glucose > ethanol	S. cerevisiae	PEG/dextran	Larsson and Mattiasson, 1984
hydrocortisone > prednisolone	Arthrobacker simplex	PEG/dextran and PEG/ReppalPES	Kaul and Mattiasson, 1986
penicillin G > 6-APA	penicillin acylase	PEG/K/HPO_4	Andersson et al. 1984

system creates a situation where the biocatalyst is temporarily immobilized in the droplets of the phase system as long as stirring is continued. The dimensions of the droplets thereby create extremely favorable conditions for mass transfer, according to Mattiasson. In an effort to stabilize such droplets a new technology was developed.

Emulsion Gelation in Aqueous Two-Phase Systems. In aqueous two-phase systems where the bottom phase is a polymer that gels at room temperature or with moderate cooling, the emulsion is formed at an elevated temperature before mild cooling takes place. The beads thus formed are very smooth and antispherical. The particle-size distribution of the particles has been studied, and it appears that the particle size profile is very much dependent upon stirring conditions and other experimental conditions, according to Mattiasson.

Mattiasson also discussed the practical and economic aspects of aqueous two-phase systems. He said that although the awareness of these systems and their ability to partition biological macromolecules has been available for several years, there has been very little practical interest in the area until a few years ago. A reluctant attitude is often encountered when presenting the technology as an alternative to other methods. Mattiasson thinks that this may partly be due to the experiences published from the spontaneous partition experiments where tedious screening for suitable phase systems was reported. However, with the introduction of affinity partitioning the partitioning of a biological macromolecule in an aqueous two-phase system has become much more predictable. The introduction of the second separator molecules, especially those of particulate character, has made this an easily operated system where there is no question of where to find the protein of interest, according to Mattiasson. Today it is regarded as a technological advantage in companies applying this separation technology, and Mattiasson said that this has led to very little publicity for practical industrial applications.

Another limiting factor has been the cost of some of the phase components used. In spontaneous partition experiments it may, in many cases, be enough to use salt/PEG sytems, where the salt is often potassium phosphate. Both these phase components are relatively cheap, but, because of their high ionic strength, they are unsuitable when performing affinity partitioning. In those cases the PEG top phase was kept and the two-phase system was formed by using a carbohydrate-based bottom phase. Most of the initial work dealt with fractionated dextran as the bottom-phase polymer. Dextran-PEG systems are now classic, and a massive amount of information concerning phase partioning of various compounds has been gathered over the years. The system seems ideal except for the price. Dextran is far too expensive to be interesting from a practical point of view, except perhaps in some rare cases when producing expensive specialty chemicals.

Therefore, in the past few years, Mattiasson and his group have developed a new starch-based polymer, Reppal-PES, with conditions very similar to dextran, except for the price. According to Mattiasson, such aqueous two-phase systems are a realistic alternative today when performing affinity partitioning. Still another limitation has been that there was no clear strategy for how to get r of the phase polymers in the final preparation. Today there are several methods available, including membrane filtration and ion-exchange chromatography. Mattiasson said that aqueous two-phase systems have proven to be extremely useful extractants with, in many cases, unique properties that meet the needs of biotechnology and that the future for these systems and the attached technology appears to be very good.

6 MEMBRANE PROCESSES

The continuous removal of ethanol from fermentation broths by pervaporation was discussed by H. Strathmann (Fraunhofer Institute for Surface Phenomena and Bioprocesses, Stuttgart, West Germany). Strathmann said that the continuous recycle of microorganisms with simultaneous removal of inhibitory products and byproducts is commonly considered as key to high conversion rates and satisfactory overall process economics in biotechnology. Another relevant economic factor is the cost of downstream processing of bioreactor constituents. The desired product is usually obtained in low concentration as part of a multicomponent mixture of different chemical and biological species. Its isolation, therefore, requires considerable capital and energy costs. Strathmann demonstrated the benefits of coupling an ethanol fermenter and a pervaporation unit. The pervaporation process combined with a solvent-selective membrane allows a particularly gentle processing of the fermentation broth, according to Strathmann. He and his group found that pervaporation across highly permeable solvent-selective membranes is the most attractive solution of the separation and concentration problems encountered in, for example, ethanol fermentation with *Saccharomyces cerevisiae*. This procedure avoids the high mechanical, thermal, or chemical stresses exerted upon microorganisms by competitive processes such as reverse osmosis, distillation, or solvent extraction and holds the greatest potential of simultaneous preconcentration of the product, according to Strathmann. It is the only solvent-selective removal process able to keep the fermentation broth in the separator under exactly identical conditions as in the fermenter. Also, the process can be operated at low temperatures, and can thus be adjusted to the optimum fermentation temperature. Since the ethanol fermentation process is exothermic, it needs to be cooled. Pervaporation takes the heat of evaporation of the permeads from the feed solution. It is, therefore, at least partially driven by the excess heat of the fermentation process.

Fundamentals of Pervaporation. Pervaporation is a membrane separation process in which a liquid mixture directly contacts one side of a thin, nonporous polymer membrane, and the permeate is removed in the vapor phase from the opposite side. The transmembrane flux is driven by a partial pressure gradient across the membrane. This can be achieved by creating a vacuum in or sweeping a carrier gas through the permeate compartment of the separator or by inducing a temperature gradient across the membrane. The selectivity of this separation process towards liquid mixtures is mainly caused by preferential sorption of solvents, according to Strathmann. Variations in diffusion coefficients can also affect the selectivity. Both phenomena are strongly related to the physico-chemical interactions between the permeating substances and the polymer. Since pervaporation is additionally selecting permeads according to their vapor pressures, salts, sugars, and higher molecular weight components are completely rejected by any membrane under pervaporation conditions. Gases will permeate pervaporation membranes according to their individual permeabilities in the selected membrane potential.

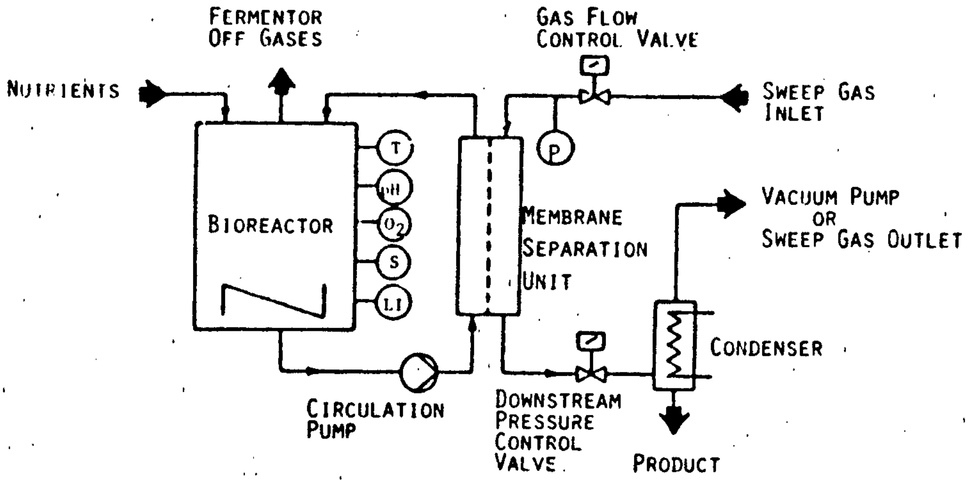

Figure 5. Process flow sheet, membrane/bioreactor system.

Selection of Appropriate Type of Membrane. The selected membrane is a composite hollow fiber-type with the active surface inside. There are two reasons for this geometry, according to Strathmann. These are: (1) there are no obstacles like spacers in the flow path of the fermentation broth so that a low plugging tendency can be expected and (2) permeate losses in the module can be controlled by varying the packing density of the fibers. The porous support is made from poly(sufone). It is coated with a solvent-selective layer of poly(dimethylsiloxane). The inner diameter of the hollow fibers can be varied from 0.5 to 2 mm. The fibers are potted into a housing similar to a tubular heat exchanger. By this design, two independent and obstacle-free flow compartments are created and both are adaptable to special process requirements. Strathmann and his group have characterized the solvent-selectivity of the synthetic membranes using a feed mixture of ethanol and water. To answer the question of fouling effects, a laboratory-scale experiment using active fermentation broth was carried out.

Design of Experimental Set-Up. An integrated membrane/bioreaction system with a fermentation volume of 350 ml was built. The hollow fiber had an area of 125 cm. Figure 5 shows the design of the process. A closed loop consisting of the bioreactor, the membrane separation unit, and a circulation pump was set up. If run without bleed, the only input into the system is the nutrient solution and the only outflow is the solvent-enriched product vapor. Product recovery rates can either be controlled by the downstream pressure control valve or the gas flow control valve. The permeated vapors are liquified in the condenser. On-line measurement of the process parameters--temperature (T), pH, oxygen concentration (O), solvent concentration (S), conductivity (Lf), and downstream pressure (p)--provides sufficient information to control the process.

Strathmann presented diagrams showing the characteristic results of vacuum pervaporation experiments obtained during 1 month of continuous fermentation of ethanol with *S. cerevisiae*. In one figure, he showed the partial flux of ethanol at a feed concentration of 5-weight-percent plotted versus permeate pressure. It showed an almost linear characteristic in the range of economically feasible vacua. Strathmann said that the ethanol recovery rate can easily be controlled by varying the downstream pressure in the given range. He also presented figures showing the long-term behavior of the pervaporation experiments. Flux and enrichment remained constant during the whole fermentation run. The average flux and selectivity at the feed concentration of 5 weight percent was found to be slightly lower than the data obtained from synthetic ethanol-water mixtures, but remained constant during the whole run. A slight fouling effect occurred immediately after contacting the membrane with the fermentation broth but no long-term fouling was observed. Strathmann said that from these results, it can be concluded that the used separator is able to accomplish the main separation tasks such as ethanol recovery, ethanol enrichment, and cell cycle.

Strathmann concluded by saying that the work he presented demonstrates the applicability of a solvent-selective pervaporation in a continuous solvent fermentation process. The experimental results showed a good stability of membrane flux and separation characteristics over long periods of time. This indicates, according to Strathmann, that the membrane polymer is completely inert towards the fermentation broth constituents. Furthermore, fouling phenomena are more or less eliminated due to the extreme smoothness of the membrane surface. Although not optimized, the membrane used in the experiments had the required properties to operate in the described mode. The membrane acts as a multifunctional element at the interface between two very distinct and basically incompatible process surroundings: the sterile fermentation broth treated under defined conditions, and the randomly contaminated environment. Strathmann listed the advantages of this concept as follows:

- Continuous product recovery
- Purification of products
- Preconcentration of products
- Sterile recycling of microorganisms and nutrients
- No thermal, mechanical, or chemical stress on the microorganisms
- Permanent control of production parameters.

The expected benefits, according to Strathmann are:

- More effective use of nutrients
- Better utilization of microorganisms
- Lower product purification costs
- Higher volumetric productivity.

7 CONCLUSION

Products of the "New Biotechnology" have successfully progressed through their research and development stages and are now becoming available as commercial products. However, it appears that a substantial number of practical problems remain to be solved. While downstream processing is one of the principal areas in which problems arise, it is crucial for the manufacture of a well-defined product of acceptable purity. This topic was addressed by several of the speakers at this intensive and focused conference. The principal aim of this conference was to discuss the influence of new materials and techniques on the isolation of new and established microbial products. It is evident from the presentations that significant progress has recently been made by scientists from different countries.

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